REMARKS

Claims 13, 24, and 29-71 are pending in the present application.

No new matter has been added. Any amendments to and/or cancellation of the claims should in no way be construed as an acquiescence to any of the Examiner's rejections and were done solely to expedite the prosecution of the application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

No additional search is required and no new issues have been raised by the arguments presented herein. Furthermore, in view of the arguments set forth herein, the issues for appeal have been reduced. It is believed that the Examiner's rejection under §103 has been obviated by the arguments presented herein. Therefore, Applicants respectfully request that the present Amendment and Response be entered.

Withdrawal of Rejection of Claims Under 35 U.S.C. § 112, Second Paragraph

Applicants gratefully acknowledge the withdrawal of the previous rejection of claims 34-37 and 44-67 under 35 U.S.C. §112, second paragraph,

Rejection of Claims 13, 24, and 29-71 Under 35 U.S.C. §103(a)

The Examiner has rejected claims 13, 24, and 29-71 under 35 U.S.C. §103(a) as being unpatentable over Fishel, *et al.* (U.S. Patent No. 6,333,153) in view of Hollingsworth, *et al.* ((1995) *Genes Dev.* 9:1728-17-39). In particular, the Examiner is of the opinion that

> [a]t the time of the claimed invention, it would have been obvious to one of ordinary skill in the art to use inhibitors of MSH5 activity identified by the methods of Fishel, as inhibitors of meiosis, chromosome synapsis and fertility, since the gene was known to facilitate these activities. It would have been further obvious to one of ordinary skill in the art to identify the inhibitors useful for contraceptive agents, since it was well known in the art that meiosis, chromosome synapsis and fertility are required for conception. Moreover, at the time of the claimed invention, one of ordinary skill in the art would have been motivated by the teachings of Hollingsworth to use the identified compounds of Fishel, as inhibitors of meiosis, fertilization and chromosome synapsis, as well as contraceptive agents.

Applicants respectfully traverse the foregoing rejection for the reasons of record and re-iterate herein that Fishel *et al.* fail to teach or suggest ***any*** association between MSH5, and contraception, fertilization, or meiosis. Nor do Fishel *et al.* teach or suggest methods for identifying candidate compounds useful for inhibiting meiosis, compounds useful as contraceptives, or compounds which prevent fertilization. Thus, the primary reference of Fishel *et al.* relied upon by the Examiner fails to teach or suggest the claimed invention.

Furthermore, Applicants respectfully submit that the secondary reference of Hollingsworth *et al.* relied on by the Examiner fails to make up for the above stated deficiencies in the primary reference of Fishel *et al.* Specifically, Hollingsworth *et al.* fail to teach or suggest that a compound which inhibits MSH5 could be used to inhibit fertilization in a subject, *i.e.,* act as a contraceptive compound. Furthermore, nowhere do Hollingsworth *et al.* teach or suggest methods for identifying candidate compounds useful for inhibiting meiosis, compounds useful as contraceptives, or compounds which prevent fertilization. Thus, Hollingsworth *et al.*, in combination with Fishel *et al.* fail to teach or suggest Applicants' invention.

In further traversal of the instant rejections, Applicants submit the following. The Examiner relies on Fishel *et al.* for teaching methods "for determining if a test composition affects (or modulates) expression of a gene encoding a MutS homologue (MSH) (col.9 line 10-15) wherein the MutS homologue is MSH5 (Col 4 line 35-40)." A review of the provisional applications to which the Fishel *et al.* patent claims priority (copies of which are submitted herewith as Appendices A, B, and C), demonstrates that the first disclosure of MSH5 as a MutS homologue for use in the screening methods of the Fishel *et al.* invention occurred on August 28, 1998, the filing date of the Fishel *et al.* patent. Specifically, U.S. Provisional Application No. 60/057,136, filed on August 28, 1997, teaches methods for making nonhuman animals nullizygous for both *Msh2* and *p53* genes and uses therof for the identification of compounds which affect tumorigeneis, apoptosis and/or aging. U.S. Provisional Application No. 60/066,977, filed on November 28, 1997, teaches methods for inducing stable associations of MutS homologues (*e.g.,* ***MSH2, MSH3, and/or MSH6*** homomers and heteromers) with a mismatched region of a DNA molecule, as well as methods to make nonhuman animals nullizygous for both *Msh2* and *p53* genes and uses therof for the identification of compounds which affect tumorigeneis, apoptosis and/or aging. Finally, U.S. Provisional Application No. 60/093,935, filed on July 23, 1998, teaches methods for influencing the activity of a MustS

homologue comprising contacting a MutS homologue (*e.g.*, ***MSH2, MSH3, and/or MSH6*** homomers and heteromers) with a MutL homologue, as well as methods to make nonhuman animals nullizygous for both *Msh2* and *p53*genes and uses therof for the identification of compounds which affect tumorigeneis, apoptosis and/or aging. Since, none of the Fishel *et al.* priority documents disclose MSH5 as a MustS homologue, the effective priority date of Fishel *et al.* for use as a prior art document in the present case is August 28, 1998.

Applicants submit herewith a declaration under 37 C.F.R. §1.131 by Drs. Winfried Edleman, Richard D. Kolodner, Jeffrey W. Pollard, and Raju S. Kucherlapati. As described in the declaration, the claimed invention was completed in this country prior to August 28, 1998, which is the effective priority date of the Fishel, *et al.* patent for the reasons set forth above.

Specifically, the manuscript attached to the declaration as Appendix D, which was authored by the inventors of the instant application, describes experiments which are the basis of the instant application, including the generation of mice carrying a null mutation in the MHS5, and the finding that mice homozygous for this mutation are viable but are sterile. The manuscript specifically discloses that MSH5 is essential for normal meiosis. Furthermore, as stated in the declaration, based on the observation that mice carrying a homozygous null mutation in the MHS5 gene are sterile and the identification of the role of MSH5 in meiosis, the inventors discovered that MSH5 can be used in screening assays for identifying a candidate compound useful as a contraceptive, a candidate compound useful for preventing fertilization, a candidate compound useful for modulating chromosome synapsis, or a candidate compound useful for modulating meiosis, as is presently claimed, prior to August 28, 1998. Thus, the claimed invention was completed in this country prior to August 28, 1998.

Accordingly, Applicants respectfully submit that the claimed invention was reduced to practice by the inventors prior to the effective date of Fishel *et al.* As such, Fishel *et al.* is not available as prior art against the present invention.

Furthermore, as set forth above, the secondary reference of Hollingsworth *et al.* fails to teach or suggest that a compound which inhibits MSH5 could be used to inhibit fertilization in a subject, *i.e.*, act as a contraceptive compound. Furthermore, nowhere do Hollingsworth *et al.* teach or suggest methods for identifying candidate compounds useful for inhibiting meiosis,

compounds useful as contraceptives, or compounds which prevent fertilization. Thus, Hollingsworth *et al.*, fail to teach or suggest Applicants' invention.

In view of the foregoing, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 13, 24, and 29-71 under 35 U.S.C. §103(a).

Previous Rejection of Claims 31 and 32 Under 35 U.S.C. §103(a)

In the Office Action dated December 31, 2002, the Examiner rejected claims 31 and 32 under 35 U.S.C. §103(a) as being unpatentable over Fishel, *et al.* (U.S. Patent No. 6,333,153) in view of Winand, *et al.* In the instant Office Action, the Examiner has stated that "the affidavits filed on January 27, 2004 under 37 C.F.R. 1.131 are not signed, and are therefore defective."

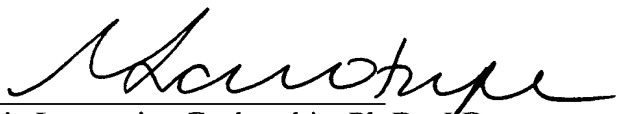
Applicants submit that the executed Declaration Under 37 C.F.R. §1.131 submitted herewith establishes that the claimed invention was reduced to practice by the inventors prior to the effective date of Fishel *et al.* and Winard *et al.* As such, Fishel *et al.* and Winard *et al.* are not available as prior art against the present invention, and accordingly, Applicants respectfully request that the Examiner reconsider and withdraw this rejection.

CONCLUSION

In view of the amendments set forth above, it is respectfully submitted that this application is in condition for allowance. If there are any remaining issues or the Examiner believes that a telephone conversation with Applicants' Attorney would be helpful in expediting prosecution of this application, the Examiner is invited to call the undersigned at (617) 227-7400.

Dated: March 30, 2005

Respectfully submitted,

By 

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Appendix A

PA 1289271

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

March 04, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: ***60/057,136***
FILING DATE: ***August 28, 1997***

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

P. R. Grant

P. R. GRANT
Certifying Officer

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Atty - # PATENT
BOX PROVISIONAL APPLICATION

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (b) (2).

Attorney Docket Number: 9855-7 (OTT-3054) Type a plus sign (+) inside this box: [+]

INVENTOR(s) APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
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anston	Aaron		Philadelphia, PA 19107
ocker	Tina		Philadelphia, PA 19107

TITLE OF INVENTION (280 characters maximum)

MOUSE CONSTRUCT NULLIZYGOUS FOR BOTH THE *Msh2* AND *p53* GENES AND METHODS OF MAKING ND USING THEREOF

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS (*check all that apply*)

Specification - *Number of Pages:* 23
Drawing(s) - *Number of Sheets:* 7
[X] Small Entity Statement
[ ] Other (*specify*):

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (*check one*)

A check or money order is enclosed to cover the Provisional filing fee.
The Commissioner is hereby authorized to charge filing fees or credit Deposit Account Number: 16-0235 (*Attach duplicate copy of this page if paying by deposit account*).
PROVISIONAL FILING FEE AMOUNT: $75.

The invention was made by an agency of the United States Government or under a contract with an agency of the U.S. vernment.

[ ] No, or [X] Yes, the name of the U.S. Government agency and the Government contract number are: NIH Grant Nos. 56542 and CA67007 and NRSA Grant No. CA73134

Respectfully submitted,

August 28, 1997
(Date)

By: Kathryn Doyle Leary
KATHRYN DOYLE LEARY, Ph.D., J.D.
Registration No. 36,317

Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

MODIFIED 10/96

19678.1

A MOUSE CONSTRUCT NULLIZYGOUS FOR BOTH THE *Msh2* AND *p53* GENES AND METHODS OF MAKING AND USE THEREOF

GOVERNMENT INTEREST

This work was supported in part by the U.S. Government (NIH grants CA56542 and CA67007 and NRSA grant CA73134), and the U.S. Government may therefore have certain rights in the invention.

FIELD OF THE INVENTION

The field of the invention is animal models for tumorigenesis, apoptosis, and aging.

BACKGROUND OF THE INVENTION

The development of transgenic animals and nullizygous animal models has provided important new avenues for the study of specific gene functions in differentiation, embryogenesis and neoplastic development (Palmiter et al., 1986, Ann. Rev. Genet. 20:465-499). Transgenic animals frequently serve as model systems for the study of various disease states and also provide an experimental system in which to test compounds for their ability to regulate disease. Nullizygous animals are similarly useful as experimental systems for the testing of compounds useful for diagnosis, treatment, or both, of disease.

Lukkarinen et al. (1997, Stroke 28:639-645) teaches that gene constructs which enable the generation of transgenic mice also enable the generation of other transgenic rodents, including rats. Similarly, nullizygous mutations in a genetic locus of an animal of one species can be replicated in an animal of another species having a genetic locus highly homologous to the first species. For example, many genetic loci

are highly homologous among mammals, and even more highly homologous among subgroups of mammals, such as among rodents.

The mutator hypothesis of tumorigenesis suggests that loss in an organism of a chromosomal stability function, a chromosomal maintenance function, or both, results in an elevated mutation rate in the organism. An elevated mutation rate hastens accumulation of the numerous mutations required for multistep carcinogenesis (Loeb, 1991, Cancer Res 51:3075-3079).

The human DNA mismatch repair (MMR) genes are highly homologous to the genes of the *Escherichia coli* MutHLS MMR system. Proteins encoded by both human and *E. coli* MMR genes contribute to genomic stability in the corresponding organism by identifying and repairing nucleotide misincorporation errors which occur during replication and exogenous DNA damage (Fishel et al., 1997, Curr. Opin. Genet. Dev. 7:105-113). Mutations in one human MMR gene, *hMsh2*, can be identified in about half of human patients afflicted with genetically linked hereditary non-polyposis colorectal cancer (HNPCC; Fishel et al., 1993, Cell 75:1027-1038 [erratum appears in 1994, Cell 77:167]; Nystrom Lahti et al., 1994, Am. J. Hum. Genet. 55:659-665). Mammalian homologues of *hMsh2* have been identified in mouse, rat, and *Drosophila* (Varlet et al., 1994, Nucl. Acids Res. 22:5723-5728; GenBank accession number X93591).

Loss of the function of p53 protein has been proposed to increase cellular hypermutability in an organism, thereby accelerating tumorigenesis, although a clear role for p53 protein in genomic instability remains controversial (Kastan et al., 1992, Cell 71:587-597; Fishel et al., 1997, Curr. Opin. Genet. Dev. 7:105-113). *p53*, the gene encoding p53 protein, is frequently mutated in a wide range of human cancers including, but not limited to, colonic tumors (Fearon et al., 1990, Cell 61:759-767). Transgenic mice nullizygous for either the *Msh2* gene or the *p53* gene are viable and susceptible to tumorigenesis (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nat. Genet. 11:64-70; Donehower et al., 1992, Nature 356:215-221; Jacks et al., 1994, Curr. Biol. 4:1-7; Purdie et al., 1994, Oncogene 9:603-609).

Although nullizygous *Msh2* mice and nullizygous *p53* mice can be used as models of carcinogenesis, the rates at which such mice develop tumors can be slower than what is desirable, particularly for large-scale screening studies involving numerous potential anti-cancer therapeutic or prophylactic compositions. What is needed is a transgenic mouse which, when exposed to a carcinogen, succumbs to tumorigenesis caused by the carcinogen more readily than does either a nullizygous *Msh2* mouse or a nullizygous *p53* mouse and which, even when not exposed to an identifiable carcinogen, succumbs to tumors more readily than does either a nullizygous *Msh2* mouse or a nullizygous *p53* mouse .

Critical unmet needs also exist for animal models of programmed cell death (apoptosis) and of aging.

SUMMARY OF THE INVENTION

The invention relates to a nonhuman animal which is nullizygous for both the *Msh2* gene and the *p53* gene. In one aspect, the nonhuman animal is a mammal, preferably a rodent, more preferably a mouse. In another aspect, the nonhuman animal has an average survival time shorter than the average survival time of a nullizygous *Msh2* animal of the same species. In still another aspect, the nonhuman animal has an average survival time shorter than the average survival time of a nullizygous *p53* animal of the same species. In yet another aspect, the nonhuman animal exhibits female-specific embryonic lethality.

The invention also relates to a method of making a nonhuman animal which is nullizygous for both the *Msh2* gene and the *p53* gene, the method comprising mating a first animal of a species, the first animal comprising at least one null allele of the *Msh2* gene of the species with a second animal of the same species, the second animal having a different gender than the first animal and comprising at least one null allele of the *p53* gene of the species. Preferably, both the first animal and the second animal are mammals; more preferably both the first animal and the second animal are rodents. Even more preferably, both the first animal and the second animal are mice.

The invention further relates to screening methods, including a method of identifying a compound which affects tumorigenesis in a mammal, a method of identifying a compound which affects apoptosis in a mammal, and a method of identifying a compound which affects the aging process in a mammal. Each of the screening methods comprises the steps of administering the compound to a first nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene, and comparing a property of the first transgenic mammal with the same property of a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered the compound, wherein a difference in the property of the first transgenic mammal compared with the property of the second transgenic mammal is an indication that the compound affects tumorigenesis, apoptosis, or the aging process, respectively in the mammal. In one aspect of the screening methods, the property is tumor incidence and a difference in this property of the first and the second mammal is an indicator that the compound affects tumorigenesis. In another aspect of the screening methods, the property is embryonic development and a difference in this property of the first and the second mammal is an indicator that the compound affects apoptosis. In a third aspect of the screening methods, the property is embryonic development and a difference in this property of the first and the second mammal is an indicator that the compound affects the aging process. Preferably, the mammal is a rodent, and even more preferably, the mammal is a mouse.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1, comprising Panels A, B, C, and D, is a series of images, each of which depicts a whole mount view of an *Msh2*$^{-/-}$*p53*$^{-/-}$ embryo at day 11.5 of development. The embryo depicted in Panel A is a male *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryo, and exhibits phenotypically normal embryonic development, relative to mice having the same genotypic background. The embryos depicted in Panels B, C, and D are female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos that are littermates of the male mouse depicted in Panel A. The female mouse embryos depicted in Panels B, C, and D exhibit

developmental arrest having a phenotype corresponding to that expected at day 9.5 of embryonic development.

Figure 2, comprising Panels A, B, C, D, E, and F, is a series of images, each of which depicts a paraffin embedded section obtained from an 11.5 day old female mouse embryo. The images in Panels A, C, and E each depict a section obtained from an 11.5 day old normal embryo. The images in Panels B, D, and F each depict a section obtained from an 11.5 day old *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryo. The sections depicted in Panels A and B are at 100× magnification and are stained with hematoxylin and eosin. Magnification of the normal embryo is of the somite region of a sagittal section. The sections depicted in Panels C and D are at 100× magnification and are chromogenically-TUNEL stained. The sections depicted Panels E and F are at 40× magnification and are fluorescently-TUNEL stained. Cells undergoing apoptosis in normal female embryos were rare; chromogenically- and fluorescently-TUNEL stained cells depicted in Panels C and E represent circumscribed apoptotic foci normally found in developing mouse embryos.

Figure 3 is a graph which depicts Kaplan-Meier survival probabilities of *Msh2*$^{-/-}$, *p53*$^{-/-}$, and *Msh2*$^{-/-}$*p53*$^{-/-}$ mice.

Figure 4 is an image of a polyacrylamide gel which was used to separate amplification transcript-length polymorphs resulting from microsatellite instability at the *D17Mit123* (CA_{26}) locus in *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. Samples were obtained from normal tissue and from thymic lymphoma tissue from each of five *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. The five individual mice are identified as m87, m132, m148, m149, and m205. Allele length alterations generated by microsatellite instability in the tumor are indicated by arrows.

DETAILED DESCRIPTION OF THE INVENTION

Transgenic mice which are nullizygous for both the *Msh2* gene and the *p53* gene have been made, and are referred to herein as *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. Other transgenic animals which are nullizygous for both the *Msh2* gene and the *p53* gene, and

which particularly include mammals, especially including rodents such as mice and rats, may be made using methods analogous to those described herein and are useful in the screening methods described herein.

The development of female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos is phenotypically arrested at approximately the 9.5 day stage, and apoptosis is induced shortly thereafter in the cells of these embryos. Male *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos are viable, but succumb to tumors significantly earlier than either *Msh2*$^{-/-}$ or *p53*$^{-/-}$ littermates (i.e. nullizygous *Msh2* mice or nullizygous *p53* mice, respectively). Furthermore, the frequency of microsatellite instability (MSI) in tumor tissue obtained from *Msh2*$^{-/-}$*p53*$^{-/-}$ mice is not significantly different than the frequency in tumor tissue obtained from *Msh2*$^{-/-}$ mice. Synergism in tumorigenesis and independent segregation of the MSI phenotype suggest that *Msh2* and *p53* are not genetically epistatic.

Msh2$^{-/-}$*p53*$^{-/-}$ mice are useful as models of disease or disorder states which cannot be identified in mice nullizygous for only one of the *Msh2* gene or the *p53* gene. Furthermore, *Msh2*$^{-/-}$*p53*$^{-/-}$ mice are useful for identifying compositions which affect the onset or progression of such a disease or disorder state. Thus, a *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse is particularly useful as a model system for studying multistep tumorigenesis.

As used herein, the term "nullizygous" refers to an animal which possesses a pair of null mutant alleles at a given genetic locus. Hence, a nullizygous *Xxx* mouse (wherein *Xxx* is any gene normally present in a mouse) does not possess a functional *Xxx* gene, whereas a wild-type mouse may possess one or two functional copies of the *Xxx* gene. To illustrate the notation used herein, the term "nullizygous *Xxx* mouse" is synonymous with the term "*Xxx*$^{-/-}$ mouse." Similarly, a "heterozygous *Xxx* mouse" has one functional *Xxx* allele and one non-functional *Xxx* allele, and is synonymous with the term "*Xxx*$^{+/-}$ mouse." A "wild type mouse" has at least one copy, and possibly two copies, of a functional *Xxx* allele, and is synonymous with the term "*Xxx*$^{+/\pm}$ mouse." A "homologous wild type mouse' has two copies of a functional *Xxx* allele, and is synonymous with the term "*Xxx*$^{+/+}$ mouse."

The materials and methods used in the studies described herein are now described.

Generation of *Msh2*$^{-/-}$*p53*$^{-/-}$ Mice

Methods for making heterozygous and nullizygous *Msh2* mice and heterozygous and nullizygous *p53* mice have been described (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nat. Genet. 11:64-70; Donehower et al., 1992, Nature 356:215-221; Jacks et al., 1994, Curr. Biol. 4:1-7; Purdie et al., 1994, Oncogene 9:603-609).

Mice heterozygous for the *Msh2* gene (i.e. *Msh2*$^{+/-}$*p53*$^{+/+}$ mice) on a mixed C57BL/6J and 129/Ola background and mice heterozygous for the *p53* gene (i.e. *Msh2*$^{+/+}$*p53*$^{+/-}$ mice) on a mixed C57BL/6J and 129/Sv were mated to produce F1 progeny heterozygous for both genes (i.e. *Msh2*$^{+/-}$*p53*$^{+/-}$ mice). Heterozygous sibling F1 progeny were intercrossed to produce progeny nullizygous for both *Msh2* and *p53* (i.e. *Msh2*$^{-/-}$*p53*$^{-/-}$ mice). Mice were genotyped using *Msh2*- and *p53*- specific PCR-based assays, using methods well known in the art.

Isolation of Genomic DNA

Mouse genomic DNA was extracted from ear-notched tissue of mice and from amniotic tissue of mouse embryos at 9.5, 11.5, or 13.5 days of development, using a QIAamp Tissue Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

PCR-based Genotyping of Mice

A three-primer assay specific for *Msh2* was carried out as described (Reitmair et al., 1995, Nat. Genet. 11:64-70). A four-primer assay specific for *p53* was carried out using 50 ng of template DNA in a 50 µl reaction mixture containing 1 unit of *Taq* polymerase (Fisher Scientific, Malvern, PA) and 100 mM each of the following primers, each of which is identified with a five digit number and the sequence of each of which is listed:

10681 (5'-GTGTTTCATTAGTTCCCCACCTTGAC-3');

10480 (5'-ATGGGAGGCTGCCAGTCCTAACCC-3');

10588 (5'-GTGGGAGGGACAAAAGTTCGAGGCC-3'); and
10930 (5'-TTTACGGAGCCCTGGCGCTCGATGT-3').
The amplification reaction involved 35 cycles of amplification (94°C, 15 seconds; 56°C, 30 seconds; 72°C, 1 minute) using a Perkin-Elmer GeneAmp 9600 thermal cycler. The wild-type primers, *10681* and *10480*, amplified a product of about 320 bp length, and the targeted allele (i.e. $p53^-$) primers, *10588* and *10930*, amplified a product of about 150 bp length.

The gender of embryos was determined using primers specific for the Y-chromosome gene as described (Sah et al., 1995, Nat. Genet. 10:175-180). The presence of the X-chromosome was confirmed separately in all cases using the following two X-chromosome specific primers to amplify the locus *DXMIT6*:
5'-ACCATTCAAATTGGCAAGG-3'; and
5'-GTGGCTCGAGTTGTTTGCAG-3'.
PCR cycling conditions were as described above for *p53* genotyping, except that the annealing temperature was 53°C, rather than 56°C. The X-chromosome specific primers amplified a product of about 210 bp in length. All PCR amplification products were resolved by electrophoresis on a 2% (w/v) agarose gel alongside a 100 bp polynucleotide ladder and were visualized by ethidium bromide staining.

Timed Pregnancies

Male and female mice that were $Msh2^{+/-}p53^{+/-}$, $Msh2^{+/-}p53^{-/-}$, or $Msh2^{-/-}p53^{+/-}$ were mated and each of the females was examined daily for the presence of a vaginal plug (an indicator of pregnancy which appears at about day 0.5 of embryo development). Pregnant females were sacrificed at 13.5 days, at 11.5 days, or at 9.5 days gestation. Embryos were dissected out from the pregnant females into Hank's Balanced Salt Solution (Gibco BRL, Grand Island, NY) under a dissecting microscope, fixed in 4% buffered formalin, and documented by photomicrography. Amnion was retrieved from each embryo, DNA extracted therefrom, and the sex and genotype of each embryo determined by PCR.

Histology

Tissue specimens were fixed in 10% (v/v) or 4% (v/v) buffered formalin and embedded in paraffin. Histological analysis was carried out on 3 μm-thick sections stained with hematoxylin and eosin (H&E).

TUNEL Assay

Paraffin-embedded tissue sections were dewaxed and rehydrated through a graded alcohol series, using methods well known in the art. Apoptotic cells and appropriate positive and negative control samples were analyzed using the *In Situ* Cell Detection Kit, AP with NBT/BCIP, manufactured by Boehringer Mannheim (Indianapolis, IN), according to the manufacturer's instructions. TUNEL-stained tissue sections were analyzed both by fluorescence and light microscopy.

Kaplan-Meier Survival

Kaplan-Meier survival probability was calculated for mice that were found dead or were sacrificed when found to be moribund. The age of the mice was calculated in days. Because no mice died in the control group, confidence limits could not be determined.

Microsatellite Instability in Lymphoid Tumors

Paired ear-notch (i.e. normal) and lymphoid tumor tissues were analyzed for microsatellite instability at five chromosomal loci: *D17Mit123*, *D10Mit2*, *D6Mit59*, *D4Mit27*, and *D3Mit203*. Microsatellite primer sequence pairs appropriate for amplification of these loci were obtained from the World Wide Web site of the Whitehead Institute for Genome Research (http://www.genome.wi.mit.edu), and were chosen to amplify fragments containing at least twenty dinucleotide repeat sequences. PCR amplifications were carried out in a total reaction volume of 25 μl, using 50 ng of DNA as template, 100 mM of each primer pair and 1 unit of *Taq* polymerase (Fisher). The amplification reaction involved 35 cycles of amplification (94°C, 15 seconds; 56°C, 30 seconds; 72°C, 1 minute). Amplified products were resolved by electrophoresis on a 6.7% (w/v) denaturing polyacrylamide gel and were visualized by silver nitrate staining of the gel.

The results of the studies described herein are now described.

Twenty-one *Msh2*$^{-/-}$*p53*$^{-/-}$ mice were generated from *Msh2*$^{+/-}$*p53*$^{+/-}$, *Msh2*$^{-/-}$*p53*$^{+/-}$, or *Msh2*$^{+/-}$*p53*$^{-/-}$ parents. When the gender of each of the twenty-one *Msh2*$^{-/-}$*p53*$^{-/-}$ mice was examined, all were determined to be male *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. The absence of female *Msh2*$^{-/-}$*p53*$^{-/-}$ offspring is highly significant ($p < 0.001$) and is unlikely to reflect the intrinsic bias for males observed in the colony corresponding to the mice, wherein the normal male:female ratio is 181:138.

The fertility of male *Msh2*$^{-/-}$*p53*$^{-/-}$ mice could not be determined, because they succumbed to tumors before they successfully mated. However, pathological examination of the testes of the male *Msh2*$^{-/-}$*p53*$^{-/-}$ mice did not reveal gross abnormalities upon autopsy, and histology revealed mature spermatogenesis in all twenty-one of the male *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. Taken together, these results suggest that *Msh2*$^{-/-}$*p53*$^{-/-}$ male mice are not likely to be sterile.

No gross morphological abnormalities were observed in *Msh2*$^{-/-}$ animals either *in utero* or post-natally (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nat. Genet. 11:64-70). In addition, the number of male and female *Msh2*$^{-/-}$ mice in the studies described herein was in accordance with the expected 1:1 ratio, which suggests that male and female nullizygous *Msh2* mice are equally viable. However, a decrease in the number of live born nullizygous *p53* mice from the expected Mendelian ratio was observed, which is qualitatively similar to previous reports, although our limited numbers did not indicate a sex bias (Sah et al., 1995, Nat. Genet. 10:175-180; Nicols et al., 1995, Nat. Genet. 10:181-187).

No female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice were found at weaning and none of thirteen one-day-old pups which were found dead in the litters of mating pairs were *Msh2*$^{-/-}$*p53*$^{-/-}$. Thus, all female embryos nullizygous for both *Msh2* and *p53* died *in utero*. To determine the point in embryonic development at which these embryos died, numerous timed pregnancies were established. Because *Msh2*$^{-/-}$*p53*$^{-/-}$ males were not available and *Msh2*$^{-/-}$*p53*$^{-/-}$ females were not viable, pairs of mice, each of which mice was a *Msh2*$^{+/-}$*p53*$^{+/-}$, *Msh2*$^{+/-}$*p53*$^{-/-}$, or *Msh2*$^{-/-}$*p53*$^{+/-}$ mouse, were mated to produce *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos. Pregnant females were sacrificed at 9.5, 11.5, and 13.5 days of gestation, the

embryos were pathologically assessed for developmental defects and their genotype and gender were determined by PCR. The results of these analyses are presented in Table 1. A total of twenty-one embryos and six resorption sites were recovered from three females at day 13.5 of gestation. Of the twenty-one 13.5 day embryos, two male *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos and no female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos were recovered, although a total of five *Msh2*$^{-/-}$*p53*$^{-/-}$embryos were statistically expected. Two 13.5 day embryos (one male *Msh2*$^{+/-}$*p53*$^{-/-}$; one female *Msh2*$^{-/-}$*p53*$^{+/-}$) displayed exencephaly, while all other 13.5 day embryos appeared normal (Sah et al., 1995, Nat. Genet. 10:175-180).

Table 1. Sex and Morphological Phenotype of Timed Post-Implantation Embryos

Days Development	Resorption Sites	# of Embryos	Embryos Typed	Female *Msh2*$^{-/-}$*p53*$^{-/-}$		Male *Msh2*$^{-/-}$*p53*$^{-/-}$	
				Nor	Abnr	Nor	Abnr
e9.5	3	30	28	3	1	2	1
e11.5	11	21	17	0	4	2	0
e13.5	6	21	21	0	0	2	0
*28	-	*96	*96	*0	*0	*21	*0

Embryos that arrested in development, that were in resorption, or that displayed gross abnormalities were classified as abnormal (Abnr), while those embryos which were not arrested in development, were not in resorption, and did not display gross abnormalities were classified as normal (Nor). Thirteen newborn pups that were found dead, none of which were *Msh2*$^{-/-}$*p53*$^{-/-}$, are not represented in this Table.

*Refers to live-born animals at twenty-eight days following birth.

Twenty-one embryos and eleven resorption sites were recovered from three pregnant females at day 11.5 of gestation. Of these, complete PCR typing results were determined for seventeen embryos and one resorption site. Five embryos were determined to be *Msh2*$^{-/-}$*p53*$^{-/-}$, although eight *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos were statistically expected. Two of the five embryos were males that appeared morphologically normal (one is depicted in Fig. 1, Panel A), and three

of the five embryos were females, all three of which had undergone developmental arrest, and all three of which are depicted in Fig. 1, Panels B, C, and D. The three female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos appeared opaque and somites were not visible. Based on the gross morphology of the three female *Msh2*$^{-/-}$ *p53*$^{-/-}$ embryos, it was calculated that they died at 9.5 days of development. The tissue from the resorption site was typed as female *Msh2*$^{-/-}$*p53*$^{-/-}$.

Thirty embryos and three resorption sites were recovered from pregnant females at day 9.5 of gestation. Twenty-eight embryos and one resorption site were successfully typed. Two embryos and a resorption site were found to be male *Msh2*$^{-/-}$*p53*$^{-/-}$, and four embryos were typed as female *Msh2*$^{-/-}$*p53*$^{-/-}$. Six *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos were statistically expected. Neither of the male *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos exhibited any gross morphological abnormality. It is likely that the male *Msh2*$^{-/-}$*p53*$^{-/-}$ resorption site represents a spontaneous abortion event. In one of the four female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos, the anterior neural tube was not closed and the heart was not seen to beat, which should occur around day 9 of development. These observations are consistent with a developmental delay that could result from late fertilization or implantation or alternatively, from a developmental abnormality that is apparent at day 9.5.

Paraffin embedded tissue sections from wildtype and *Msh2*$^{-/-}$ *p53*$^{-/-}$ female embryos, as depicted in Fig. 2, from *Msh2*$^{-/-}$ embryos, and from *p53*$^{-/-}$ embryos were examined at day 11.5 and at day 13.5. While the wildtype, *Msh2*$^{-/-}$, and *p53*$^{-/-}$ embryos had clearly distinguished developmental features at day 11.5, the arrested *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryos contained noncohesive cells without preservation of embryonal tissue structures. In addition, H&E stained *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryonic tissue sections appeared to contain an large number of "blebbed" structures typical of apoptotic cells. Furthermore, loss of nuclear hematoxylin stain typical for necrosis was not observed in H&E stained *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryonic tissue sections (Fig. 2, Panel B).

TUNEL staining was performed on the paraffin embedded tissue sections (Fig. 2, Panels C-F). Although wildtype (Fig. 2, Panels C and E), *Msh2*$^{-/-}$, and *p53*$^{-/-}$ embryos displayed circumscribed foci of apoptotic cells characteristic of normal embryonal development, *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryos displayed global catastrophic apoptosis (Fig. 2, Panels D and F). Furthermore, fluorescence TUNEL staining of *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryos revealed a speckled intracellular patterning characteristic of fragmented chromatin (Fig. 2, Panel F). It was estimated that between about 60% and about 90% of cells in *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryos were undergoing visible apoptosis, as assessed by H&E and TUNEL staining.

Kaplan-Meier survival analysis was performed on a cohort of ninety-six mice, the data for which analysis are graphically depicted in Fig. 3. *Msh2*$^{-/-}$*p53*$^{-/-}$ mice began to die of generalized lymphomas at day 53 after birth and all twenty-one *Msh2*$^{-/-}$*p53*$^{-/-}$ mice were dead within four months of birth. In contrast, only 18% (eight of forty-four) of *Msh2*$^{-/-}$ littermates and 71% (five of seven) of *p53*$^{-/-}$ littermates were dead at the time the mice were analyzed. Thus, *Msh2*$^{-/-}$*p53*$^{-/-}$ mice had a significantly ($p<0.001$) reduced median survival time of 73 days compared with the median survival time of either *Msh2*$^{-/-}$ mice (i.e. 200 days) or *p53*$^{-/-}$ mice (i.e. 149 days). Furthermore, all twenty-four wild-type (i.e. *Msh2*$^{+/\pm}$*p53*$^{+/\pm}$) littermates were alive after approximately ten months. These results indicate that *Msh2* and *p53* null mutations cooperatively promote tumorigenesis. *p53* has also been shown to cooperate with a variety of other genes in mouse tumorigenesis models (Blyth et al., 1995, Oncogene 10:1717-1723; Williams et al., 1994, Cold Spring Harbor Symp. Quant. Biol. 59:449-457; Williams et al., 1994, Cell 79:329-339; Donehower et al., 1995, Genes Dev. 9:882-895; Nacht et al., 1996, Genes Dev. 10:2055-2066). However, as is apparent from Fig. 3, the effect on tumor-related death of having dual null mutations of *Msh2* and *p53* is greater than the sum of the effects of having a single null mutation in *Msh2* or *p53* alone. Thus, the *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse

described herein has a phenotype which is significantly different from a mere combination of the phenotype of a *Msh2*$^{-/-}$ mouse and the phenotype of a *p53*$^{-/-}$ mouse.

Pathological examination of tumors showed that all twenty-one *Msh2*$^{-/-}$*p53*$^{-/-}$ mice developed highly aggressive generalized lymphomas involving major organs. In addition, a pleomorphic sarcoma in the flank, a malignant fibrous histiocytoma of the neck, and a tubular adenoma of the small intestine were observed, while other epithelial neoplasms were not detected. The tumor spectrum of *Msh2*$^{-/-}$ and *p53*$^{-/-}$ mice appeared similar to previous observations (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nat. Genet. 11:64-70; Donehower et al., 1992, Nature 356:215-221; Jacks et al., 1994, Curr. Biol. 4:1-7; Purdie et al., 1994, Oncogene 9:603-609). The tumor spectrum of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice differs significantly from the tumor spectrum of either *Msh2*$^{-/-}$ or *p53*$^{-/-}$ mice. Thus, *Msh2*$^{-/-}$*p53*$^{-/-}$ mice have utility different from that of either *Msh2*$^{-/-}$ or *p53*$^{-/-}$ mice.

Normal and tumor tissues obtained from individual *Msh2*$^{-/-}$*p53*$^{-/-}$ mice were examined for microsatellite instability at five loci: *D17Mit123*, *D10Mit2*, *D6Mit59*, *D4Mit27*, and *D3Mit203* (Fig. 4). The results of these MSI studies are presented in Table 2. The frequency of MSI in tumor tissues obtained from *Msh2*$^{-/-}$ mice was not significantly different ($p>0.05$) the frequency of MSI in tumor tissues obtained from *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. Microsatellite instability was not observed in lymphomatous tumor tissue obtained from the seven *p53*$^{-/-}$ mice examined. The observation that *Msh2*$^{-/-}$*p53*$^{-/-}$ mice developed earlier onset of tumor-related disease, combined with the observed separate segregation of the MSI phenotype with the *Msh2* allele, suggests that *Msh2* and *p53* are not genetically epistatic.

Table 2. The Frequency of Microsatellite Instability in *p53*$^{-/-}$, *Msh2*$^{-/-}$, and *Msh2*$^{-/-}$*p53*$^{-/-}$ Mice.

Genotype Tumor / Normal Pairs	Tumors Examined (n)	MSI at ≥1 Locus	MSI at ≥2 Loci	MSI at ≥3 Loci
p53$^{-/-}$	7	0 (0%)	0 (0%)	0 (0%)
Msh2$^{-/-}$	8	6 (75%)	4 (50%)	3 (38%)
**Msh2*$^{-/-}$*p53*$^{-/-}$	21	17 (81%)	14 (67%)	12 (57%)

*Because female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice died during embryonic development, this refers to only male *Msh2*$^{-/-}$*p53*$^{-/-}$ mice.

It is remarkable that female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos underwent global developmental arrest and that widespread apoptosis of the cells of such embryos occurred around day 9.5 of development. That these embryos underwent implantation and gastrulation strongly suggests that they are capable of executing the earlier stages of embryogenesis. The arrested phenotype is reminiscent of that described for a small proportion of female *p53*$^{-/-}$ mice (Sah et al., 1995, Nat. Genet. 10:175-180). However, unlike *p53*$^{-/-}$ mice, no normal female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice or embryos were observed beyond 9.5 days of embryonic development. This observation supports the conclusion that the female embryonic lethality of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice is highly penetrant. In addition, none of the female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos displayed the exencephaly that characterized the *p53*$^{-/-}$ mice (Sah et al., 1995, Nat. Genet. 10:175-180). Furthermore, while there was no difference in apoptosis observed in developing *p53*$^{-/-}$ mouse embryos, global catastrophic apoptosis was clearly observed in all the *Msh2*$^{-/-}$*p53*$^{-/-}$ female mouse embryos examined. These results suggest that female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice succumb at an earlier stage and by an entirely different pathology than *p53*$^{-/-}$ mice.

Without being bound to any particular theory, the lethality observed in female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos is consistent with the

following explanation. In the female embryonic lineage, dosage compensation is achieved by random X chromosome inactivation around the time of gastrulation, at which time intense embryonic cellular proliferation and apoptosis promote embryonic differentiation (Lyon, 1961, Nature 190:372-373; Rastan, 1994, Curr. Opin. Genet. Dev. 4:292-297; Theiler, 1972, In: The House Mouse Development and Normal Stages from Fertilization to 4 Weeks of Age, Springer-Verlag, New York, p. 168). The global apoptotic effect need not occur coincidentally with X chromosome inactivation. The full effect of dysregulation may only become apparent after a number of cell divisions when the embryo undergoes a further burst of proliferation during embryonic 'turning' between 8 and 9.5 days.

It has been shown that the inactivated X chromosome replicates late in S phase (Taylor, 1960, J. Biophys. Biochem. Cytol. 7:455-464; Tagaki, 1974, Exp. Cell Res. 86:127-135). In addition, cells deficient in *p53* have been shown to be defective for damage-induced G_1/S checkpoint arrest, and cells that are deficient in MMR have been shown to be deficient for damage-induced G_2/M checkpoint arrest (Baker et al., 1990, Science 249:912-915; Diller et al., 1990, Mol. Cell. Biol. 10:5772-5781; Lin et al., 1992, Proc. Natl. Acad. Sci. USA 89:9210-9214; Hawn et al., 1995, Canc. Res. 55:3721-3725; Marra et al., 1996, Oncogene 13:2189-2196). Thus, female-specific *Msh2*$^{-/-}$*p53*$^{-/-}$ embryo lethality may result from dysregulation of damage-induced arrest checkpoint control, wherein such dysregulation is caused by a deficiency of both *p53* and *Msh2*, and whereby such dysregulation results in an inability of *Msh2*$^{-/-}$*p53*$^{-/-}$ cells to arrest cell division and repair damage introduced into the late replicating inactive X chromosome. Such damage could take the form of nonreplicated regions or chromosomal fragments that have resulted from inappropriate cell division prior to the completion of inactive X chromosome replication. Fragmented, reactivated, or otherwise altered inactive X chromosomes may then lead to global catastrophic cellular failure,

developmental arrest, and apoptosis. Furthermore, the observation that the highest levels of p53 mRNA are detected in wild-type embryos between 9 and 11 days of development suggests an important role for p53 protein within this time frame (Rogel et al., 1985, Mol. Cell. Biol. 5:2851-2855).

Screening Methods

A standard screening procedure is now described which is useful for determining the tumorigenetic potential of a compound. *Msh2*$^{-/-}$ *p53*$^{-/-}$ mice are generated as described herein. While this procedures is described with respect to particular protocols and mice, it will be appreciated that the screening procedure described should not be construed to limit the invention in any way.

A predetermined amount of the compound is administered to the *Msh2*$^{-/-}$ *p53*$^{-/-}$ mouse by any practical means. The method of administration of the compound is not critical. By way of example, the compound may be administered orally, intraperitoneally, intravenously, topically, intramuscularly, or via a pulmonary route.

To reduce any potential for bias, the study is blinded. A first investigator treats all mice with compound(s) and identifiably marks or cages the transgenic mice, so that the nature of the treatments will not be known to a second investigator, who performs all tumor counts, weighing, and general observations.

Following administration of the compound, the *Msh2*$^{-/-}$ *p53*$^{-/-}$ mouse, each *Msh2*$^{-/-}$ *p53*$^{-/-}$ mouse is observed for about four months. Each mouse is examined approximately daily. Every week, each mouse is weighed, observed for any clinically-relevant symptoms, and the number and extent of tumors are assessed.

After observations are completed, the rate of tumor incidence and the tumor yield are determined for each group of *Msh2*$^{-/-}$ *p53*$^{-/-}$ mice to which the compound was applied. A higher or lower rate of tumor incidence or

a higher or lower tumor yield for a group of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice to which the compound was applied, compared with the levels of tumor incidence and tumor yield for a group of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice to which the compound was not applied, is an indication that the compound affects tumorigenesis.

Preferably, groups of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice are used, with each mouse in a group being treated identically. Also preferred are studies in which one of at least three different dose levels of the compound are applied to the mice in each of at least three corresponding groups of transgenic mice. It is preferred, where possible, to demonstrate a statistically significant difference ($P < 0.05$) between the average rate of tumor incidence or the average tumor yield for the first dose level and the average rate of tumor incidence or the average tumor yield for the third dose level.

Msh2$^{-/-}$*p53*$^{-/-}$ mice may also be used to identify the ability of a compound to affect apoptosis. This assay is performed identically to the tumorigenesis assay described herein, except that the compound is administered to *Msh2*$^{-/-}$*p53*$^{-/-}$ mice *in utero*, and the embryonic development of female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice is assessed, rather than tumor incidence or yield. A difference between embryonic development in female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos to which the compound was administered and embryonic development in female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos to which the compound was not administered is an indication that the compound affects apoptosis.

Msh2$^{-/-}$*p53*$^{-/-}$ mice may also be used to identify the ability of a compound to affect the aging process. This assay is performed identically to the apoptosis assay described herein, except that a difference between embryonic development in female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos to which the compound was administered and embryonic development in female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos to which the compound was not administered is an indication that the compound affects the aging process.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

What is claimed is:

1. A nonhuman animal which is nullizygous for both the *Msh2* gene and the *p53* gene.

2. The nonhuman animal of claim 1, wherein the nonhuman animal is a mammal.

3. The nonhuman animal of claim 1, wherein the nonhuman animal is a rodent.

4. The nonhuman animal of claim 1, wherein the nonhuman animal is a mouse.

5. The nonhuman animal of claim 1, wherein the nonhuman animal has an average survival time shorter than the average survival time of a nullizygous *Msh2* animal of the same species.

6. The nonhuman animal of claim 1, wherein the nonhuman animal has an average survival time shorter than the average survival time of a nullizygous *p53* animal of the same species.

5. The nonhuman animal of claim 1, wherein the nonhuman animal exhibits female-specific embryonic lethality.

6. A method of making a nonhuman animal which is nullizygous for both the *Msh2* gene and the *p53* gene, the method comprising mating a first animal of a species, the first animal having a gender and comprising at least one null allele of the *Msh2* gene of the species with a second animal of the species, the second animal having a different gender than the first animal and comprising at least one null allele of the *p53* gene of the species.

7. The method of claim 6, wherein the first animal and the second animal are mammals.

8. The method of claim 6, wherein the first animal and the second animal are rodents.

9. The method of claim 6, wherein the first animal and the second animal are mice.

10. A method of identifying a compound which affects tumorigenesis in a mammal comprising

administering the compound to a first nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene, and

comparing the tumor incidence in the first transgenic mammal with the tumor incidence in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered the compound, wherein a difference in tumor incidence in the first transgenic mammal compared with the tumor incidence in the second transgenic mammal is an indication that the compound affects tumorigenesis in the mammal.

11. The method of claim 10, wherein the first nonhuman mammal and the second nonhuman mammal are rodents.

12. The method of claim 10, wherein the first nonhuman mammal and the second nonhuman mammal are mice.

13. A method of identifying a compound which affects apoptosis in a mammal comprising

administering the compound *in utero* to a first nonhuman mammal embryo which is nullizygous for both the *Msh2* gene and the *p53* gene, and

comparing embryonic development in the first transgenic mammal embryo with embryonic development in a second nonhuman animal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered the compound, wherein a difference in embryonic development of the first transgenic mammal compared with embryonic development of the second transgenic mammal is an indication that the compound affects apoptosis in the mammal.

14. The method of claim 13, wherein the first nonhuman animal and the second nonhuman mammal are rodents.

15. The method of claim 13, wherein the first nonhuman mammal and the second nonhuman mammal are mice.

16. A method of identifying a compound which affects the aging process in a mammal comprising

administering the compound *in utero* to a first nonhuman mammal embryo which is nullizygous for both the *Msh2* gene and the *p53* gene, and

comparing embryonic development in the first transgenic mammal embryo with embryonic development in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered the compound, wherein a difference in embryonic development of the first transgenic mammal compared with embryonic development of the second transgenic mammal is an indication that the compound affects the aging process in the mammal.

17. The method of claim 16, wherein the first nonhuman mammal and the second nonhuman mammal are rodents.

18. The method of claim 16, wherein the first nonhuman mammal and the second nonhuman mammal are mice.

ABSTRACT

The invention relates to nonhuman animals, particularly mice, which are nullizygous for the *Msh2* and *p53* genes, which animals exhibit abnormal rates of tumorigenesis, apoptosis, female embryonic lethality, and DNA microsatellite instability. Methods of using the animals, particularly mice, to identify compounds which affect tumorigenesis, apoptosis, or the aging process are described.

Attorney's Docket No. 9855-7 (OTT-3054)

Applicant or Patentee: **Richard Fishel, Aaron Cranston and Tina Bocker**
Application or Patent No.: **Not Yet Assigned**
Filed or Issued: **Herewith**
For: **A Mouse Construct Nullizygous For Both The *Msh2* And *p53* Genes And Methods Of Making and Use Thereof**

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

NAME OF ORGANIZATION: **Thomas Jefferson University**
ADDRESS OF ORGANIZATION: 1020 Walnut Street
Philadelphia, PA 10107

TYPE OF ORGANIZATION:

[X] University or other institution of higher education.
[ ] Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)).
[ ] Nonprofit scientific or educational under statute of state of the United States of America.
Name of State _____
Citation of Statute _____
[ ] Would qualify as tax exempt under Internal Revenue Code (26 USC 501(a) and 501(c)(3) if located in the United States of America.
[ ] Would qualify as nonprofit scientific or educational under statute of state of United States of America if located in the United States of America.
Name of State _____
Citation of Statute _____

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, with regard to the invention entitled **A Mouse Construct Nullizygous For Both The *msh2* and *p53* Genes And Methods Of Making And Use Thereof** by inventor(s) **Richard Fishel, *et al.*** described in

[X] the specification filed herewith.
[ ] Application No. _____
Filed _____
[ ] Patent No. _____
Issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27).

FULL NAME _____
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FULL NAME _____
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I acknowledge the duty to file, in this application or patent, notification of any change in the status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

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TITLE IN ORGANIZATION Director of the Office of Technology Transfer
ADDRESS OF PERSON SIGNING 1020 Locust Street
Philadelphia, PA 19107-6799

SIGNATURE Abram Goldfinger DATE: 8/28/97

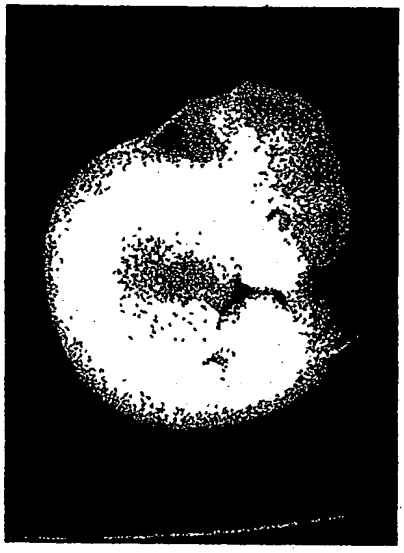


FIGURE 1A

FIGURE 2B

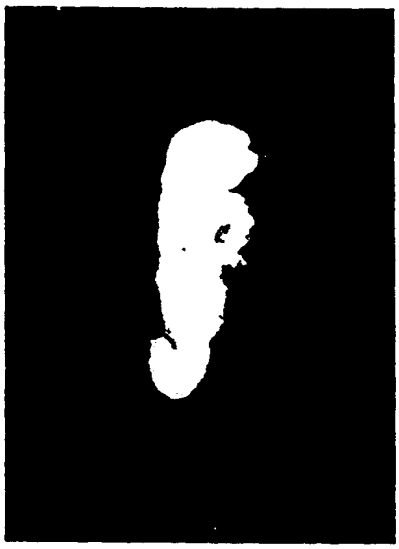


FIGURE 1C

FIGURE 1D

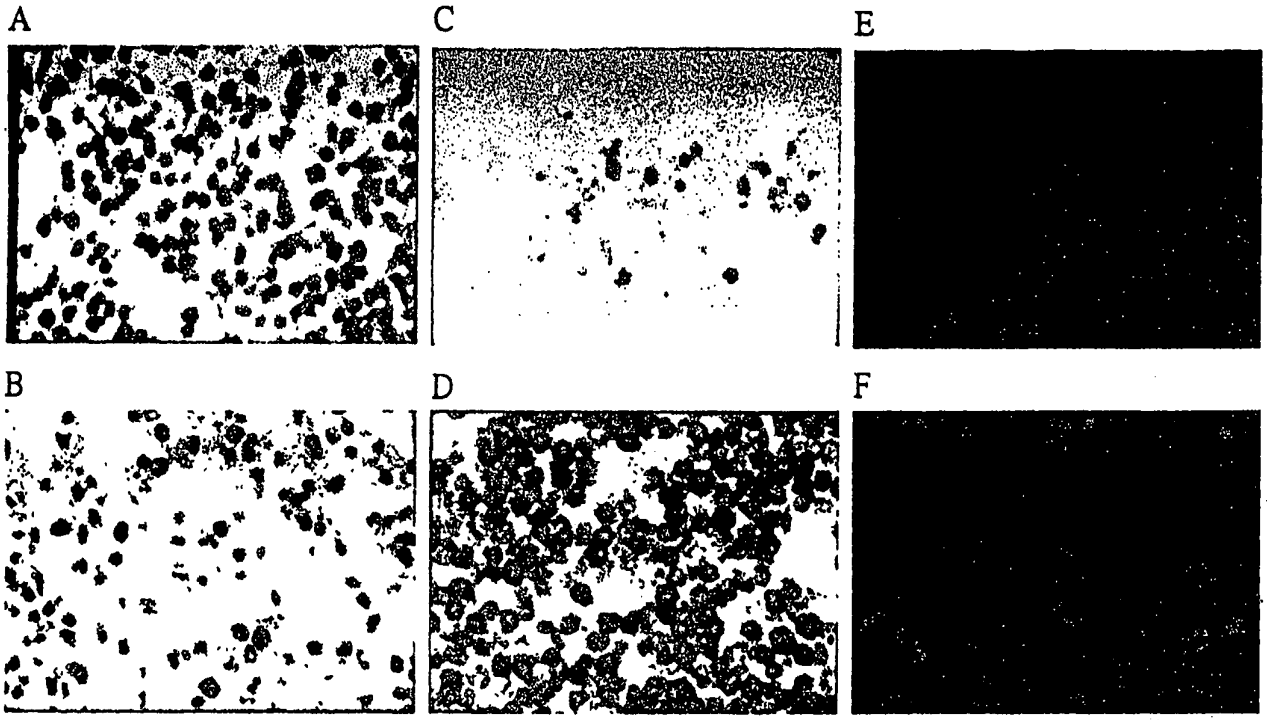


FIGURE 2

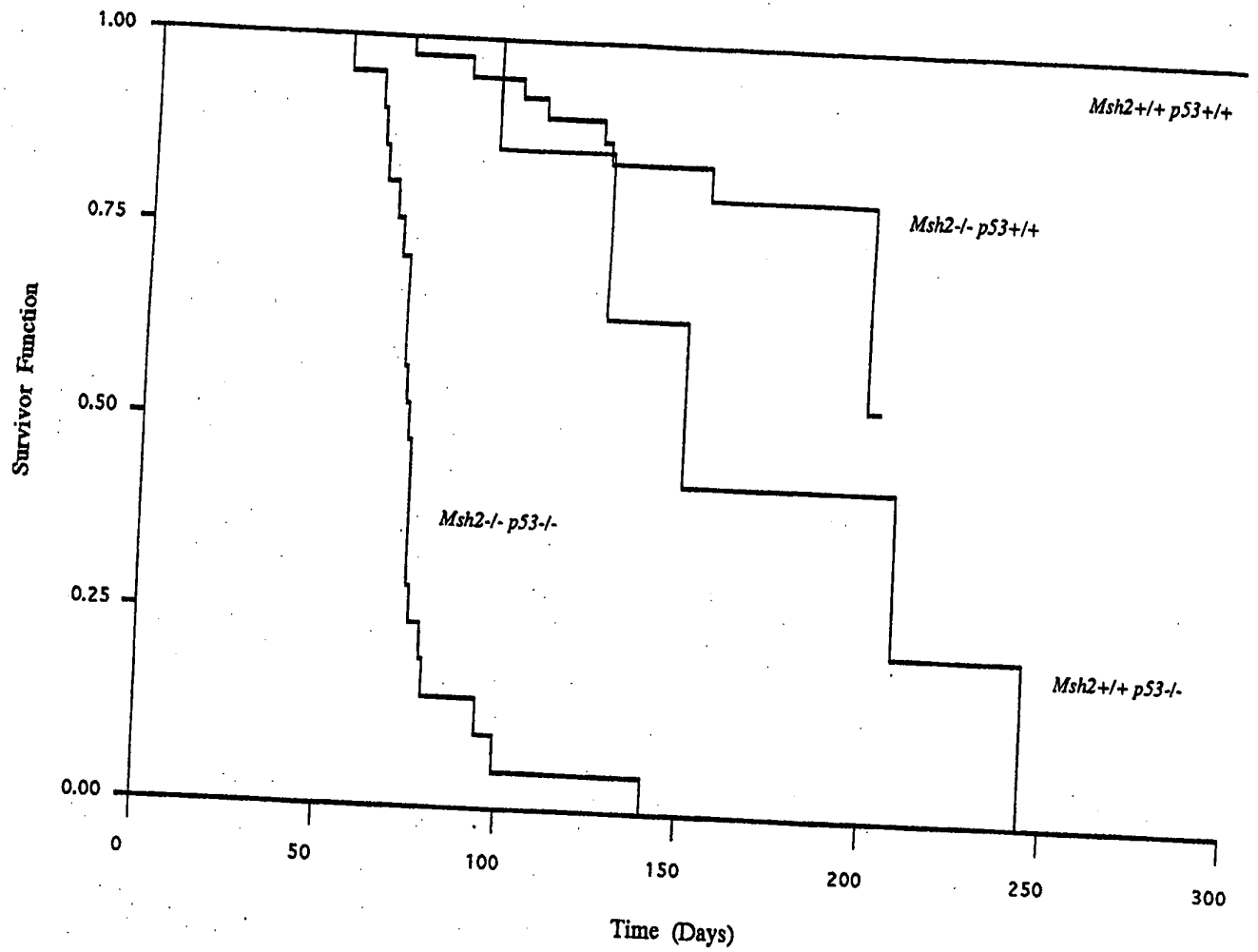
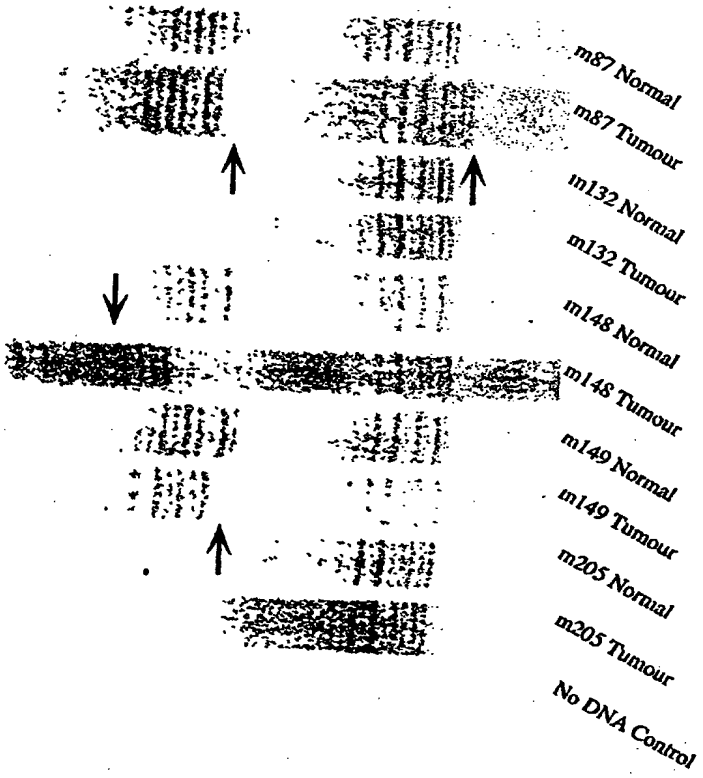

1.00
0.75
0.50
0.25
0.00
Survivor Function
Msh2+/+ p53+/+
Msh2-/- p53+/+
Msh2-/- p53-/-
Msh2+/+ p53-/-
0
50
100
150
200
250
300
Time (Days)

FIGURE 3

FIGURE 4


m87 Normal
m87 Tumour
m132 Normal
m132 Tumour
m148 Normal
m148 Tumour
m149 Normal
m149 Tumour
m205 Normal
m205 Tumour
No DNA Control

PA 1289271

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March 03, 2005

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APPLICATION NUMBER: ***60/066,977***
FILING DATE: ***November 28, 1997***

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

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"EXPRESS MAIL" MAILING LABEL NO.: EM607113856US

PATENT
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (b) (2).

Attorney Docket Number: 9855-6 (OTT-3026) Type a plus sign (+) inside this box: [+]

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TITLE OF INVENTION (280 characters maximum)

ADP-ACTIVATION OF DNA MISMATCH BINDING PROTEINS

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ENCLOSED APPLICATION PARTS (*check all that apply*)

[X] Specification - *Number of Pages:* *83*
[ ] Drawing(s) - *Number of Sheets:* *17 (informal)*
[X] Small Entity Statement
[ ] Other (*specify*):

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (*check one*)

[ ] A check or money order is enclosed to cover the Provisional filing fee.
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[X] PROVISIONAL FILING FEE AMOUNT: **$75.**

The invention was made by an agency of the United States Government or under a contract with an agency of the U.S. Government.

[ ] No, or [X] Yes, the name of the U.S. Government agency and the Government contract number are: NIH Grant Nos. CA56542 and CA 67007 and NRSA grant CA73134

Respectfully submitted,

November 28, 1997
(Date)

By: Kathryn Doyle Leary
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[X] Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

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PATENT
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET
(LISTING FOR ADDITIONAL INVENTORS)

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (b) (2).

Attorney Docket Number: 9855-6 (OTT-3026) Type a plus sign (+) inside this box: [+]

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ADP-ACTIVATION OF DNA MISMATCH BINDING PROTEINS

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PROVISIONAL APPLICATION FILING ONLY

MODIFIED 10/96

9855-6
OTT-3026

ADP-ACTIVATION OF DNA MISMATCH BINDING PROTEINS

GOVERNMENT SUPPORT

This research was supported in part by U.S. Government funds (NIH grants numbers CA56542 and CA67007 and NRSA grant CA73134), and the U.S. Government may therefore have certain rights in the invention.

FIELD OF THE INVENTION

The field of the invention is DNA mismatch protein binding, including animal models for tumorigenesis, apoptosis, and aging.

BACKGROUND OF THE INVENTION

The most widely accepted model for DNA post-replication mismatch repair is based largely on the model of the DNA adenine methylation (Dam)-Instructed pathway of *Escherichia coli* proposed by Modrich (1986, Basic Life Sci. 38:303-310; Modrich, 1987, Ann. Rev. Biochem. 56:435-466; Modrich, 1989, J. Biol. Chem. 264:6597-6600; Modrich, 1991, Annu. Rev. Genet. 25:229-253; Modrich et al., 1996, Annu. Rev. Biochem. 65:101-133). According to this model, the MutS protein recognizes and binds mismatched nucleotides resulting from polymerase misincorporation errors to form a MutS-DNA product (Su et al., 1986, Proc. Natl. Acad. Sci., U.S.A. 83:5057-5061; Su et al., 1988, J. Biol. Chem. 263:6829-6835). MutS mismatch binding is followed by the interaction of MutL protein with the MutS-DNA product (Grilley et al., 1990, Mutat. Res. 236:253-267), which accelerates ATP-dependent translocation of the MutS-MutL complex (Allen et al., 1997, EMBO Journal 16:4467-4476) to a hemimethylated GATC Dam site to which MutH protein is bound (Welsh et al., 1987, J. Biol. Chem. 262:15624-15629; Au et al., 1992, J. Biol. Chem.

267:12142-12148). The MutS-MutL complex stimulates an intrinsic endonuclease activity of MutH protein, which cleaves the non-methylated (i.e. more recently replicated) DNA strand (Welsh et al., 1987, J. Biol. Chem. 262:15624-15629; Lahue et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:1482-1486; Su et al., 1989, Genome 31:104-111; Cooper et al., 1993, J. Biol. Chem. 268:11823-11829; Grilley et al., 1993, J. Biol. Chem. 268:11830-11837). Strand cleavage enables one of three single-stranded exonucleases of *E. coli* (RecJ, ExoI, ExoVII) to degrade the non-methylated strand, which can then be resynthesized by the *E. coli* PolIII holoenzyme complex (Lahue et al., 1989, Science 245:160-164). The net result is a strand-specific mismatch repair event.

Many genetic studies performed using *E. coli* support this interpretation. For example bacteria having a mutated *mutH*, *mutL*, or *mutS* gene exhibit a mutator phenotype that is presumed to be the result of the increased probability of misincorporation errors leading to mutations (Demerec et al., 1957, Bacterial Genetics, Carnegie Inst. Wash. Yearbook 370:390-406; Miyake, 1960, Genetics 45:755-762; Siegel et al., 1967, J. Bacteriol. 94:38-47; Hill, 1970, Mutat. Res. 9:341-344). However, not all predictions arising from the *E. coli* Dam-instructed model agree with experimental results. For example, bacteria having a mutation in each of the *recJ*, *exoI*, and *exoVII* genes do not exhibit a mutator phenotype, suggesting that other exonuclease(s) or mechanism(s) are involved in the mismatch repair process.

Homologs of the procaryotic MutS and MutL proteins have been identified in eukaryotes (Fishel et al., 1993, Cell 75:1027-1038; Prolla et al., 1994, Science 265:1091-1093; Bronner et al., 1994, Nature 368:258-261). MutH analogs appear to exist only in gram-negative bacteria.

Multiple MutS and MutL homologs have been identified in yeast and human cells which individually participate in such diverse activities as nuclear and organelle mismatch repair as well as distinct meiotic functions (Fishel et al., 1997, Curr. Opin. Genet. Dev. 7:105-113). Germ-line mutations of the human MutS and MutL Homologs, hMSH2, hMLH1, and hPMS2, have been found to be associated with

the common cancer predisposition syndrome, hereditary non-polyposis colorectal cancer (HNPCC; Bronner et al., 1994, Nature 368:258-261; Fishel et al., 1993, Cell 75:1027-1038). Yeast and human MutS and MutL homologs exist primarily as heterodimeric proteins. Yeast MSH2 protein has been found to be associated with MSH3 or MSH6, and yeast MLH1 has been found to be associated with PMS1. Human hMSH2 protein has been found to be associated with hMSH3 or hMSH6 (also designated GTBP or p160 by some authors), and human hMLH1 has been found to be associated with hPMS2 (Li et al., 1995, Proc. Natl. Acad., Sci. U.S.A. 92: 1950-1954; Prolla et al., 1994, Science 265:1091-1093; Drummond et al., 1995, Science 268:1909-1912; Marsischky et al., 1996, Genes & Development 10:407-420; Acharya et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:13629-13634). Furthermore, MSH2/MSH3 and MSH2/MSH6 protein complexes appear to possess overlapping and redundant mismatch binding activities (Acharya et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:13629-13634; Risinger et al., 1996, Nat. Genet. 14:102-105).

Classification of MutS and MutL homologs is based on the presence in the proteins of highly conserved regions of amino acid identity. The most highly conserved region among MutS homologs includes approximately 150 amino acids which comprise a helix-turn-helix domain associated with a Walker A adenine-nucleotide and magnesium binding motif (Walker et al., 1982, EMBO J. 1:945-951). This adenine nucleotide binding domain constitutes more than 80% of the identifiable homology between MutS homologs (Fishel et al., 1997, Curr. Opin. Genet. Dev. 7:105-113). Both purified bacterial MutS homologs and purified yeast MutS homologs possess an intrinsic low-level ATPase activity (Haber et al., 1991, EMBO. J. 10:2707-2715; Chi et al., 1994, J. Biol. Chem. 269: 29993-29997; Chi et al., 1994, J. Biol. Chem. 269:29984-29992; Alani et al., 1997, Mol. Cell Biol. 1 7: 2436-2447). This ATPase activity is likely to be important for the function of MutS homologs, as indicated by the fact that mutation of conserved amino acid residues in the adenine nucleotide binding domain results in a dominant mutator phenotype in both bacteria and yeast (Haber et al., 1991, EMBO. J. 10:2707-2715; Wu et al., 1994, J. Bacteriol

176:5393-5400; Alani et al., 1997, Mol. Cell Biol. 1 7: 2436-2447). A central role for the adenine nucleotide binding domain is consistent with the ATP-dependent translocation model of mismatch repair proposed by Modrich and colleagues (Allen et al., 1997, EMBO Journal 16:4467-4476).

Genetic and biochemical studies of the human mismatch repair process indicate that it is similar to bacterial mismatch repair, except that the physiologically relevant mechanism for directing strand specificity is unknown (Miller et al., 1976, Proc. Natl. Acad. Sci. U.S.A. 73:3073-3077; Glazer et al., 1987, Mol. Cell. Biol., 7:218-224; Holmes et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:5837-5841; Thomas et al., 1991, J. Biol. Chem. 266:3744-3751; Fang et al., 1993, J. Biol. Chem. 268:11838-11844; Longley et al., 1997, J. Biol. Chem. 272: 10917-10921). Purified hMSH2 protein binds mismatched nucleotides and DNA lesions (Fishel et al., 1994, Science 266:1403-1405; Fishel et al., 1994, Cancer Res. 54:5539-5542; Mello et al., 1996, Chemistry & Biology 3:579-589), and the specificity and affinity of that recognition is enhanced by association of hMSH2 with hMSH3 or hMSH6 (Drummond et al., 1995; Acharya et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:13629-13634; Palombo et al., 1996, Curr. Biol. 6:1181-1184).

The development of transgenic animals and nullizygous animal models has provided important new avenues for the study of specific gene functions in differentiation, embryogenesis and neoplastic development (Palmiter et al., 1986, Ann. Rev. Genet. 20:465-499). Transgenic animals frequently serve as model systems for the study of various disease states and also provide an experimental system in which to test compounds for their ability to regulate disease. Nullizygous animals are similarly useful as experimental systems for the testing of compounds useful for diagnosis, treatment, or both, of disease.

Lukkarinen et al. (1997, Stroke 28:639-645) teaches that gene constructs which enable the generation of transgenic mice also enable the generation of other transgenic rodents, including rats. Similarly, nullizygous mutations in a genetic locus of an animal of one species can be replicated in an animal of another species having a

genetic locus highly homologous to the first species. For example, many genetic loci are highly homologous among mammals, and even more highly homologous among subgroups of mammals, such as among rodents.

The mutator hypothesis of tumorigenesis suggests that loss in an organism of a chromosomal stability function, a chromosomal maintenance function, or both, results in an elevated mutation rate in the organism. An elevated mutation rate hastens accumulation of the numerous mutations required for multistep carcinogenesis (Loeb, 1991, Cancer Res 51:3075-3079).

Loss of the function of p53 protein has been proposed to increase cellular hypermutability in an organism, thereby accelerating tumorigenesis, although a clear role for p53 protein in genomic instability remains controversial (Kastan et al., 1992, Cell 71:587-597; Fishel et al., 1997, Curr. Opin. Genet. Dev. 7:105-113). *p53*, the gene encoding p53 protein, is frequently mutated in a wide range of human cancers including, but not limited to, colonic tumors (Fearon et al., 1990, Cell 61:759-767). Transgenic mice nullizygous for either the *Msh2* gene or the *p53* gene are viable and susceptible to tumorigenesis (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nat. Genet. 11:64-70; Donehower et al., 1992, Nature 356:215-221; Jacks et al., 1994, Curr. Biol. 4:1-7; Purdie et al., 1994, Oncogene 9:603-609).

Although nullizygous *Msh2* mice and nullizygous *p53* mice can be used as models of carcinogenesis, the rates at which such mice develop tumors can be slower than what is desirable, particularly for large-scale screening studies involving numerous potential anti-cancer therapeutic or prophylactic compositions. What is needed is a transgenic mouse which, when exposed to a carcinogen, succumbs to tumorigenesis caused by the carcinogen more readily than does either a nullizygous *Msh2* mouse or a nullizygous *p53* mouse and which, even when not exposed to an identifiable carcinogen, succumbs to tumors more readily than does either a nullizygous *Msh2* mouse or a nullizygous *p53* mouse .

Critical unmet needs also exist for animal models of programmed cell death (apoptosis) and of aging.

In addition, there is a critical unmet need for the identification and isolation of DNA fragments comprising mismatch nucleotides and the use thereof. The present invention satisfies these needs.

SUMMARY OF THE INVENTION

The invention relates to a method of binding a MutS homolog to a mismatched region of a duplex DNA molecule, the method comprising contacting the homolog with the duplex DNA molecule in the presence of a solution comprising an ADP molecule, whereby the homolog binds to the mismatched region.

In a preferred embodiment, the homolog is selected from the group consisting of the *E. coli* MutS protein, an hMSH2 protein dimer, a complex comprising an hMSH2 protein molecule and either an hMSH3 protein molecule or an hMSH6 protein molecule, a yeast MSH2 protein dimer, a complex comprising a yeast MSH2 protein molecule and either a yeast MSH3 protein molecule or a yeast MSH6 protein molecule, a dimer of a rat homolog of hMSH2, a dimer of a *Xenopus* homolog of hMSH2, a dimer of a *Drosophila* homolog of hMSH2, a dimer of a murine homolog of hMSH2, and a complex comprising a murine homolog of hMSH2 and either a murine homolog of hMSH3 or a murine homolog of hMSH6. More preferably, the homolog comprises a complex comprising an hMSH2 protein molecule and an hMSH6 protein molecule.

In other preferred embodiments, the solution comprises at least about 20 micromolar ADP and the solution comprises less than about 5 micromolar ATP.

In another preferred embodiment, the solution further comprises ATP and the ratio of the concentration of ADP in the solution to the ratio of the concentration of ATP in the solution is greater than about two.

Also preferably, the solution is substantially free of ATP.

In yet other preferred embodiments, the duplex DNA molecule is suspended in the solution and the homolog is suspended in the solution.

The invention also relates to a method of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules. The method comprises providing a MutS homolog and a solution comprising an ADP molecule and the population of DNA molecules, whereby the homolog binds to the mismatched region, and segregating the homolog from the population, whereby the duplex DNA molecule is segregated from the population.

In a preferred embodiment, the homolog is bound to a solid support and the process of segregating the homolog from the population comprises rinsing the solid support with a solution which does not comprise the population.

In another preferred embodiment, the population comprises a plurality of cDNA molecules, wherein each of the cDNA molecules is made by reverse transcription of an RNA molecule obtained from an organism.

In yet another preferred embodiment, the organism is a mammal, preferably, a human.

In another preferred embodiment, the method further comprises contacting the homolog with a solution comprising an ATP molecule after segregating the homolog from the population, whereby the mismatched region dissociates from the homolog.

There is also included in the invention a method of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence. The method comprises annealing a first single-stranded DNA molecule and a second single-stranded DNA molecule to form a duplex DNA molecule, wherein the first molecule has the sample sequence and wherein the second molecule has a nucleotide sequence which is complementary to the reference sequence, whereby if there is a difference between the sample sequence and the reference sequence then the duplex DNA molecule has a mismatched region, thereafter contacting the duplex DNA molecule and a MutS homolog in the presence of a solution comprising an ADP molecule, whereby the homolog binds specifically to the duplex DNA molecule if the duplex DNA molecule comprises the mismatched region, and thereafter determining

whether the homolog binds specifically to the duplex DNA molecule, whereby specific binding of the homolog to the duplex DNA molecule is an indication that there is a difference between the sample nucleotide sequence and the reference nucleotide sequence.

In a preferred embodiment of this aspect of the invention, the sample nucleotide sequence comprises the sequence of a region of a gene obtained from a first organism and the reference nucleotide sequence comprises the sequence of the region obtained from a second organism.

In other preferred embodiments, the first organism and the second organism are the same species, preferably, a human.

In yet another preferred embodiment, the sample nucleotide sequence comprises the sequence of a region of a gene obtained from an organism and the reference nucleotide sequence comprises a consensus nucleotide sequence of the region. Preferably, the gene is the human *msh2* gene.

Also included in the invention is a kit for separating a mismatched duplex DNA molecule from a population of molecules, the kit comprising a MutS homolog, a linker for binding the homolog to a solid support, and an ADP molecule.

There is also provided in the invention a method of detecting a predisposition of a mammal to carcinogenesis. The method comprises annealing a first single-stranded DNA molecule and a second single-stranded DNA molecule to form a duplex DNA molecule, wherein the first molecule has the nucleotide sequence of a region of a mammalian gene associated with carcinogenesis and wherein the second molecule has a nucleotide sequence which is complementary to the consensus nucleotide sequence of the region, whereby if there is a sequence difference between the first molecule and the second molecule then the duplex DNA molecule has at least one mismatched region, thereafter contacting the duplex DNA molecule and a MutS homolog in the presence of a solution comprising an ADP molecule, whereby the homolog binds to the duplex DNA molecule if the duplex DNA molecule comprises a mismatched region, and thereafter determining whether the homolog binds to the

duplex DNA molecule, whereby binding of the homolog to the duplex DNA molecule is an indication of the predisposition of the mammal to carcinogenesis.

In a preferred embodiment of this aspect of the invention, the mammalian gene associated with carcinogenesis is selected from the group consisting of the *msh2* gene, the *msh3* gene, the *msh6* gene, the *mlh1* gene, the *pms2* gene, the *brca1* gene, the *brca2* gene, the *pten* gene, and the *p53* gene. Preferably, the mammalian gene is the human *msh2* gene and the predisposition of a mammal for carcinogenesis is predisposition of a human for hereditary non-polyposis colorectal cancer.

There is also provided a method of fractionating a population of duplex DNA molecules. The method comprises contacting the population with a MutS homolog in the presence of ADP, whereby the homolog is capable of binding to a duplex DNA molecule of the population if the duplex DNA molecule comprises a mismatched region, segregating the homolog from the population, and contacting the homolog with a dissociation solution comprising an ATP molecule, whereby the duplex DNA molecule dissociates from the homolog, whereby the population is fractionated.

In addition, there is provided a method of selectively amplifying at least one mismatched duplex DNA molecule of a population of duplex DNA molecules. The method comprises contacting the population with a MutS homolog in the presence of a solution comprising an ADP molecule, whereby the homolog binds to the at least one mismatched duplex DNA molecule, thereafter segregating the homolog from the population, whereby each the at least one mismatched duplex DNA molecule is segregated from the population, and thereafter amplifying each the at least one mismatched duplex DNA molecule using a PCR technique, whereby each of the at least one mismatched duplex DNA molecule is selectively amplified.

Also included is a method of detecting the presence of a genetic polymorphism in the genome of an animal. The method comprises amplifying a region of each of two copies of a gene of the animal to yield an amplified first copy and an

amplified second copy, thereafter mixing and denaturing each of the first copy and the second copy to yield a first mixture of nucleic acids comprising a first sense copy, a first antisense copy, a second sense copy, and a second antisense copy, thereafter annealing the nucleic acids in the first mixture to yield a second mixture of nucleic acids comprising the first copy, the second copy, a first sense-second antisense duplex DNA molecule, and a second sense-first antisense duplex DNA molecule, whereby if the first sense copy and the second antisense copy are not completely complementary then the first sense-second antisense duplex DNA molecule will have a first mismatched region, and whereby if the second sense copy and the first antisense copy are not completely complementary then the second sense-first antisense duplex DNA molecule will have a second mismatched region, thereafter contacting the second mixture with a MutS homolog in the presence of a solution comprising an ADP molecule, whereby the homolog is capable of binding to either of the first mismatched region or the second mismatched region, thereafter segregating the homolog from the second mixture, and thereafter detecting the presence or absence of either of the first sense-second antisense duplex DNA molecule or the second sense-first antisense duplex DNA molecule, whereby the presence of either of the first sense-second antisense duplex DNA molecule or the second sense-first antisense duplex DNA molecule is an indication that the animal has a genetic polymorphism.

The invention further includes a composition for segregating a mismatched duplex DNA molecule from a population of molecules. The composition comprises a MutS homolog bound to a solid support, wherein the support is in liquid contact with a solution comprising an ADP molecule.

Further included is a kit for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence. The kit comprises a pair of primers adjacent the region for amplifying the region, a duplex DNA molecule having the reference nucleotide sequence, a solid support, a MutS homolog, a linker for binding the MutS homolog to the solid support, and an ADP molecule.

In addition, the invention includes a nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene. Preferably, the mammal is a rodent; more preferably, a mouse.

In preferred embodiments, the mammal has an average survival time shorter than the average survival time of a nullizygous *Msh2* mammal of the same species and the mammal has an average survival time shorter than the average survival time of a nullizygous *p53* mammal of the same species. In addition, the mammal exhibits female-specific embryonic lethality.

There is also included a method of making a nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene. The method comprises mating a first mammal of a species, the first mammal comprising at least one null allele of the *Msh2* gene of the species with a second mammal of the species, the second mammal comprising at least one null allele of the *p53* gene of the species.

In addition, there is provided a method of identifying a compound which affects tumorigenesis in a mammal comprising administering the compound to a first nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene, and comparing tumor incidence in the first transgenic mammal with tumor incidence in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered the compound, wherein a difference in tumor incidence in the first transgenic mammal compared with tumor incidence in the second transgenic mammal is an indication that the compound affects tumorigenesis in the mammal.

Further incldued is a method of identifying a compound which affects apoptosis in a mammal comprising administering the compound *in utero* to a first nonhuman mammal embryo which is nullizygous for both the *Msh2* gene and the *p53* gene, and comparing embryonic development in the first transgenic mammal embryo with embryonic development in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered the compound, wherein a difference in embryonic development of the first transgenic mammal

compared with embryonic development of the second transgenic mammal is an indication that the compound affects apoptosis in the mammal.

The invention also includes a method of identifying a compound which affects the aging process in a mammal comprising administering the compound *in utero* to a first nonhuman mammal embryo which is nullizygous for both the *Msh2* gene and the *p53* gene, and comparing embryonic development in the first transgenic mammal embryo with embryonic development in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered the compound, wherein a difference in embryonic development of the first transgenic mammal compared with embryonic development of the second transgenic mammal is an indication that the compound affects the aging process in the mammal.

In addition, there is provided a cell line which is nullizygous for both the *Msh2* gene and the *p53* gene, wherein the cell line is made by culturing a cell obtained from a nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an image which depicts proteins detected in preparations containing the hMSH2/hMSH6 complex. Proteins were separated by SDS-PAGE. Molecular weight markers are shown on the left of the Figure and in teh lane designated "MWM." Lane 1 depicts proteins present in a crude extract which was obtained from the insect cells described herein. Lane 2 depicts proteins present in peak fractions obtained from the nickel affinity column purification step described herein. Lane 3 depicts proteins present in peak fractions obtained from the PBE anion exchange column purification step described herein. Proteins were stained using Coomassie Brilliant blue dye. The positions of protein bands corresponding to hMSH2 and to hMSH6 are indicated.

Figure 2, comprising Figures 2A, 2B, 2C, 2D, 2E, and 2F, depict binding of hMSH2/hMSH6 protein complex to mismatched and non-mismatched

DNA. Figure 2A is an image of the results of a gel mobility shift assay performed using the G/T-mismatched 81-base pair DNA substrate described herein. The concentrations of complex are indicated along the top of the image. The position of the S-shifted electrophoretic band is indicated by "S". Figure 2E is a graph which depicts the relationship between the concentration of complex and the amount of product corresponding to the S-shifted electrophoretic band in Figure 2A, as assessed using a phosphoimaging device. Figure 2B is an image of the results of a gel mobility shift assay performed using the homologous 81-base pair DNA substrate described herein. The concentrations of complex are indicated along the top of the image. The position of the NS-shifted electrophoretic band is indicated by "NS". Figure 2F is a graph which depicts the relationship between the concentration of complex and the amount of product corresponding to the NS-shifted electrophoretic band in Figure 2B, as assessed using a phosphoimaging device. Figure 2C is an image which depicts the results of a DNase footprint assay performed using the G/T-mismatched 81-base pair DNA substrate described herein. The concentrations of complex are indicated along the top of the image. The position of the G residue of the G/T-mismatched substrate is indicated by "G", and the approximate region of the substrate protected from DNase cleavage by the complex is indicated by a vertical line. Figure 2D is an image which depicts the results of a DNase footprint assay performed using the homologous 81-base pair DNA substrate described herein. The concentrations of complex are indicated along the top of the image. The position of the G/C base pair corresponding to the G/T-mismatched base pair of the mismatched substrate is indicated by "G".

Figure 3, comprising Figures 3A, 3B, and 3C, depicts kinetic characterization data for the ATPase activity of the hMSH2/hMSH6 protein complex. Figure 3A is a graph which depicts the extent of ATP hydrolysis after 30 minutes as a function of selected concentrations of the complex, as assessed in the presence or absence of mismatched DNA, in the presence or absence of homologous DNA, and in the presence of magnesium chloride. Figure 3B is an image which depicts the results of TLC analysis of the products of the ATPase activity of the complex in the presence

of mismatched DNA. Lane 1 depicts the products formed in the ATPase reaction mixture in the absence of the complex. Lane 2 depicts the products formed in the ATPase reaction mixture in the presence of 60 nanomolar complex. The positions of AMP, ADP, and ATP are indicated to the right of the lane. Figure 3C is a double-reciprocal plot of steady-state ATPase activity at selected ATP concentrations, as assessed in the presence or absence of homologous DNA and in the presence or absence of mismatched DNA. The inset depicts a magnification of the portion of the plot near the intercepts of the 1/V plots with the horizontal and vertical axes.

Figure 4, comprising Figures 4A, 4B, 4C, and 4D, depicts the results of gel mobility shift assays used to assess the ability of various adenine nucleotides to convert the S-shifted electrophoretic band to the NS-shifted electrophoretic band. Figure 4A is an image of an assay in which the product corresponding to the S-shifted electrophoretic band was incubated in the presence of ATP at the concentration listed along the top of the image. Figure 4B is an image of an assay in which the product corresponding to the S-shifted electrophoretic band was incubated in the presence of adenosine-5'-O-3'-thiotriphosphate (ATP-γ-S) at the concentration listed along the top of the image. Figure 4C is an image of an assay in which the product corresponding to the S-shifted electrophoretic band was incubated in the presence of ADP at the concentration listed along the top of the image. In Figures 4A, 4B, and 4C, "-" indicates that no complex was included in the assay mixture. Figure 4D is a graph which depicts quantitated results obtained using the results depicted in Figures 4A, 4B, and 4C, as assessed using a phosphoimaging device.

Figure 5 is a bar graph which depicts the effect of selected nucleotides, deoxynucleotides, and nucleotide analogs on G/T mismatch binding by the complex, relative to the degree of binding observed in the absence of a (deoxy)nucleotide or analog. The effect of each indicated (deoxy)nucleotide or analog was assessed at 25 micromolar (left bar of each pair) and at 250 micromolar (right bar of each pair).

Figure 6, comprising Figures 6A, 6B, 6C, 6D, and 6E, depicts the effects of ATP hydrolysis or ADP binding by the hMSH2/hMSH6 protein complex on

mismatched DNA binding. Figure 6A is an image which depicts the results of gel mobility shift assays performed in the presence or absence of 15 micromolar ATP. Magnesium chloride was added at the time designated "0", and samples of the assay mixture were collected at the indicated times (in minutes). The binding reaction in each mixture was halted by the addition of 5 millimolar EDTA. Figure 6B is an image which depicts the results of gel mobility shift assays performed in the presence or absence of 15 micromolar ATP-γ-S. Magnesium chloride was added at the time designated "0", and samples of the assay mixture were collected at the indicated times (in minutes). The binding reaction in each mixture was halted by addition of 5 millimolar EDTA. Figure 6C is a graph of the results depicted in Figures 6A and 6B, as assessed using a phosphoimaging device. Figure 6D is an image which depicts the results of gel mobility shift assays performed in the presence of the indicated (in millimolar) concentrations of ATP or ADP or both. In each of Figures 6A, 6B, and 6D, the left-most lane depicts results obtained using an assay mixture which did not contain the complex, and S-shifted and NS-shifted electrophoretic bands are indicated by "S" and "NS", respectively. Figure 6E is a graph of the results depicted in Figure 6D, as assessed using a phosphoimaging device.

Figure 7 comprises Figures 7A, 7B, 7C, and 7D. Figure A is a diagram which depicts the mechanism by which the hMSH2/hMSH6 complex is switched to the "ON" form, which is capable of binding mismatched DNA, from the "OFF" form, which has a much lower affinity for mismatched DNA. Switching from the "OFF" to the "ON" form involves hydrolysis of ATP bound to the complex, which hydrolysis is catalyzed by the complex. Switching from the "ON" to the "OFF" form involves displacement of ADP bound to the complex by an ATP molecule. Figure 7B is a graph which depicts the results obtained in the assays described herein for detecting the rate of a single round of ATP hydrolysis by the complex. Figure 7C is a graph which depicts the results obtained in assays described herein for detecting the rate of a single round of ATP hydrolysis by the complex in the presence of selected amounts of mismatched DNA. Figure 7D is a graph which depicts the results obtained in assays

described herein for detecting the exchange of ADP for ATP in the presence or absence of mismatched DNA. "%ADP bound" means the percentage of ADP that was initially bound to the complex which remained bound at the time indicated in the graph.

Figure 8, comprising Figures 8A, 8B, 8C, and 8D, depicts the results of experiments performed to assess the effects of ATP, homologous DNA, or both, on the dissociation of the hMSH2/hMSH6 complex from DNA. Figure 8A is an image of the results obtained from gel mobility shift assays in which complex-bound mismatched DNA was incubated with ATP for the time indicated in the image. Figure 8B is an image of the results obtained from gel mobility shift assays in which complex-bound mismatched DNA was incubated with ATP and a 400-fold excess of homologous DNA for the time indicated in the image. Figure 8C is an image of the results obtained from gel mobility shift assays in which complex-bound mismatched DNA was incubated with a 400-fold excess of homologous DNA for the time indicated in the image. Figure 8D is an image of the results obtained from gel mobility shift assays in which the complex was incubated with homoduplex DNA probe for fifteen minutes at 37°C (Lane A), the assay mixture was cooled to 4°C, and a 1,100-fold excess of unlabeled competitor homoduplex DNA was added (Lane B). In each of Figure 8A, 8B, 8C, and 8D, "-" indicates assay mixtures which did not comprise the complex.

Figure 9 is a diagram which depicts the model of the hMSH2/hMSH6 protein complex binding to mismatched DNA described herein. The ADP-bound form of the complex, which is shown in the center of the diagram, is competent to bind mismatched DNA, as shown at the bottom of the diagram. Mismatched DNA-bound complex may be displaced therefrom by displacing the ADP molecule bound to the complex with an ATP molecule, which yields the ATP-bound form of the complex, which is shown at the top of the diagram. The ATP-bound form of the complex may be converted to the ADP-bound form by hydrolysis of the complex-bound ATP molecule, catalyzed by ATPase activity of the complex.

Figure 10 lists the nucleotide sequence of single nucleotide chains of the 39- and 81-base pair DNA substrates described herein (SEQ ID NOS:1-6).

Figure 11, comprising Panels A, B, C, and D, is a series of images, each of which depicts a whole mount view of an *Msh2*$^{-/-}$*p53*$^{-/-}$ embryo at day 11.5 of development. The embryo depicted in Panel A is a male *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryo, and exhibits phenotypically normal embryonic development, relative to mice having the same genotypic background. The embryos depicted in Panels B, C, and D are female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos that are littermates of the male mouse depicted in Panel A. The female mouse embryos depicted in Panels B, C, and D exhibit developmental arrest having a phenotype corresponding to that expected at day 9.5 of embryonic development.

Figure 12, comprising Panels A, B, C, D, E, and F, is a series of images, each of which depicts a paraffin embedded section obtained from an 11.5 day old female mouse embryo. The images in Panels A, C, and E each depict a section obtained from an 11.5 day old normal embryo. The images in Panels B, D, and F each depict a section obtained from an 11.5 day old *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryo. The sections depicted in Panels A and B are at 100× magnification and are stained with hematoxylin and eosin. Magnification of the normal embryo is of the somite region of a sagittal section. The sections depicted in Panels C and D are at 100× magnification and are chromogenically-TUNEL stained. The sections depicted Panels E and F are at 40× magnification and are fluorescently-TUNEL stained. Cells undergoing apoptosis in normal female embryos were rare; chromogenically- and fluorescently-TUNEL stained cells depicted in Panels C and E represent circumscribed apoptotic foci normally found in developing mouse embryos.

Figure 13 is a graph which depicts Kaplan-Meier survival probabilities of *Msh2*$^{-/-}$, *p53*$^{-/-}$, and *Msh2*$^{-/-}$*p53*$^{-/-}$ mice.

Figure 14 is an image of a polyacrylamide gel which was used to separate amplification transcript-length polymorphs resulting from microsatellite instability at the *D17Mit123* (CA_{26}) locus in *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. Samples were obtained from normal tissue and from thymic lymphoma tissue from each of five *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. The five individual mice are identified as m87, m132, m148, m149, and m205.

Allele length alterations generated by microsatellite instability in the tumor are indicated by arrows.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of inducing stable association of a MutS homolog with a mismatched region of a DNA molecule. The methods of the invention are based on the discovery that binding of an ADP molecule to a MutS homolog stabilizes the association of the ADP-bound homolog with a mismatched region of a DNA molecule.

Homologs of the *E. coli* MutS protein are known to be involved in DNA mismatch repair, including the MutS homologs encoded by the human *hMSH2*, *hMSH3*, and *hMSH6* genes, designated hMSH2, hMSH3, and hMSH6, respectively. hMSH2 is capable of forming a protein complex with either hMSH3 or hMSH6 and these two complexes, designated the hMSH2/hMSH3 protein complex and the hMSH2/hMSH6 protein complex, respectively, are capable of binding to mismatched DNA. A MutS homolog can be caused to dissociate from a mismatched region of a DNA molecule by contacting the homolog with ATP. It is believed that contacting the homolog with ATP caused an ATP-hydrolysis-dependent translocation of the homolog along the mismatched DNA strand. However, in order that the association of a MutS homolog with a mismatched region of a DNA molecule be exploited as a useful phenomenon, it is necessary that the complex which is formed is stabilized. According to the discovery of the present invention, a means of stabilizing a MutS homolog/DNA complex is provided. The ability to provide a stable MutS homolog/DNA complex facilitates the exploitation of the formation of this complex as a useful entity as described herein.

The methods of the invention include a method of binding a MutS homolog to a mismatched region of a duplex DNA molecule, comprising contacting the

MutS homolog and the duplex DNA molecule in the presence of a solution comprising an ADP molecule, whereby the MutS homolog binds to the mismatched region.

In the method of the invention of binding a MutS homolog to a mismatched region of a duplex DNA molecule, the MutS homolog may be any MutS homolog which is presently known to be or is discovered to be involved in DNA mismatch repair. Thus, by way of example, the MutS homolog useful in the methods, products, and compositions of the invention may be the *E. coli* MutS protein, an hMSH2 protein dimer, a complex comprising an hMSH2 protein molecule and either an hMSH3 protein molecule or an hMSH6 protein molecule, a yeast MSH2 protein dimer, a complex comprising a yeast MSH2 protein molecule and either a yeast MSH3 protein molecule or a yeast MSH6 protein molecule, a dimer of a rat homolog of hMSH2 (e.g. GenBank accession number X93591), a dimer of a *Xenopus* homolog of hMSH2 (Varlet et al., 1994, Nucl. Acids Res. 22:5723-5728), a dimer of a *Drosophila* homolog of hMSH2 (e.g. GenBank accession number U17893), a dimer of a murine homolog of hMSH2 (e.g. GenBank accession number X93591, Varlet et al., 1994, Nucl. Acids Res. 22:5723-5728), a complex comprising a murine homolog of hMSH2 and either a murine homolog of hMSH3 (e.g. Rep-3; Linton et al., 1989, Mol. Cell. Biol. 9:3058-3072; Smith et al., 1990, Mol. Cell. Biol. 10:6003-6012) or a murine homolog of hMSH6 (e.g. Gen Bank accession number U42190), and the like. It is understood that, given the high degree of similarity among mammalian MutS homologs (Fishel et al., 1997, Curr. Op. Genet. Develop. 7:105-113), a dimer of any mammalian hMSH2 homolog can be used in the methods of the invention. Similarly, any complex comprising any mammalian hMSH2 homolog and either any mammalian hMSH3 homolog or any mammalian hMSH6 homolog can be used in the methods of the invention.

In the method of the invention of binding a MutS homolog to a mismatched region of a duplex DNA molecule, the DNA molecule may be any duplex DNA molecule having a mismatched region. By way of example, the DNA molecule may be a linear DNA molecule, a circularized DNA molecule such as a plasmid or a

viral genome, a chromosome, a cDNA generated by reverse transcription of an RNA molecule, a PCR primer, a PCR product, a complex formed between a single-stranded DNA probe and another single-stranded DNA molecule, and the like. The mismatched region may be any region of a duplex DNA molecule in which the two DNA strands of the molecule are not completely complementary. By way of example, the mismatched region may comprise one or more pairs of mismatched nucleotides in an otherwise complementary region of a duplex DNA molecule, a region of a duplex DNA molecule wherein a thymine dimer exists on one DNA strand of the molecule, a region of a duplex DNA molecule comprising a nucleotide which has been covalently modified by an agent capable of reacting with a nucleotide, such as cisplatin, a region of a duplex DNA molecule which comprises an alkyl-O-6-methyl guanine residue, a region of a duplex DNA molecule which comprises a single stranded loop of one or more nucleotides, a region of a duplex DNA molecule which comprises a pyrimidine dimer, and the like.

While any amount of ADP can be used in the method of the invention of binding a MutS homolog to a mismatched region of a duplex DNA molecule, it is preferred that the homolog be contacted with the DNA molecule in the presence of a solution comprising at least about 20 micromolar ADP. As described with greater particularity in Example 1, ATP displaces ADP from the MutS homolog. Thus, it is important either that the concentration of ATP in the solution be minimized, for example by maintaining the concentration of ATP lower than about 5 micromolar, or that the ratio of the concentration of ADP in the solution to the concentration of ATP in the solution be greater than a minimum value, such as about two. Preferably, the solution is substantially free of ATP, by which is meant that the concentration of ATP in the solution is lower than 5 micromolar; preferably, the concentration of ATP in the solution is 1 micromolar or lower.

In the method of the invention of binding a MutS homolog to a mismatched region of a duplex DNA molecule, either or both of the MutS homolog and the DNA molecule may be suspended in the ADP-containing solution. Alternately,

either or both of the MutS homolog or the DNA molecule may be fixed to a surface which contacts the ADP-containing solution. Where the MutS homolog is fixed to a first surface, such as a latex bead or the like, and the DNA molecule is fixed to a second surface, such as a glass slide, a microwell plate, or the like, it is necessary that the first and second surfaces be moveable with respect to one another, such that the MutS homolog and the DNA molecule are capable of contacting one another in the presence of the ADP-containing solution.

The MutS homolog may be bound to a surface using any known method for attaching a protein to a surface. For example the MutS homolog may be bound to a surface by way of an antibody which is covalently bound to the surface and which has a variable region which specifically binds to the MutS homolog. By way of example, an antibody which specifically binds to hMSH2 such as the antibody described by Kinzler et al. (PCT application WO96/41192, published December 19, 1996) may be used to bind an hMSH2 protein dimer or a complex comprising an hMSH2 protein molecule and either an hMSH3 protein molecule or an hMSH6 protein molecule to a surface to which the antibody is fixed. Methods of fixing an antibody to a surface have been described in the art (e.g. Harlow et al., 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York).

The duplex DNA molecule may be bound to a surface using any known method for attaching a nucleic acid to a surface. By way of example, the nucleic acid may be covalently linked to a biotin molecule and the surface may be linked to or coated with a streptavidin molecule, whereby the streptavidin molecule is capable of binding the biotin molecule, thereby linking the nucleic acid to the surface.

The methods of the invention also include a method of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules. This method comprises contacting a MutS homolog and the population of DNA molecules in the presence of a solution comprising an ADP molecule and then segregating the homolog from the population. As described herein, the MutS homolog is capable of binding to the mismatched region of the DNA molecule, whereupon the

DNA molecule may be physically manipulated by manipulating the MutS homolog or a solid support linked to the homolog.

The method used to segregate the MutS homolog from the population of DNA molecules in the method may be any method by which the concentration of the homolog in at least a portion of the homolog-population mixture can be changed relative to the concentration of the population. Such methods include, but are not limited to, linking the homolog to a surface, such as a polystyrene surface, which contacts the mixture, precipitating the homolog from the mixture, precipitating DNA molecules which are not bound to the homolog from the mixture, linking each DNA molecule of the population to a surface which contacts the mixture, and hydrolyzing or otherwise degrading the DNA molecules of the population after contacting the homolog with the population.

By way of example, the method of the invention of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules may be performed by binding a MutS homolog to a solid support, contacting the solid support with the population of DNA molecules, and rinsing the solid support with a solution which does not comprise the population of DNA molecules. In this example, a mismatched DNA molecule in the population of DNA molecules binds to the MutS homolog and thereby becomes linked to the solid support, and the mismatched DNA molecule is segregated from the other DNA molecules of the population by rinsing the support with a solution which carries the other DNA molecules away from the solid support. Thus, according to this example, the mismatched DNA molecule is physically separated from the other DNA molecules of the population.

It is not necessary that the just-described method result in separation of the DNA molecule having a mismatched region from the population given that the molecule and the population are contained in different containers at the conclusion of the method. By way of example, it is sufficient in the optical affinity biosensor system (OABS) described elsewhere herein that a DNA molecule comprising a mismatched region associate with the detector surface of the OABS and that non-mismatched DNA

molecules do not associate with the detector surface of the OABS. Thus, for example, in OABS methods for detection of mismatched DNA molecules, a MutS homolog may be associated with the detector surface of the OABS, whereby a DNA molecule having a mismatched region binds to the homolog in the presence of ADP and is detected, and whereby a DNA molecule not having a mismatched region does not bind appreciably to the homolog and is not detected.

The DNA molecules of the population of DNA molecules of the method of the invention of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules may be any DNA molecules, so long as at least one of the DNA molecules is a duplex DNA molecule having a mismatched region. By way of example, each of the DNA molecules of the population may be a cDNA molecule generated by reverse transcription of an RNA molecule obtained from an organism such as an animal, a mammal, or a human.

In a variation of the method of the invention of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules, the method further comprises contacting the MutS homolog with a solution comprising an ATP molecule after segregating the homolog from the population of DNA molecules, whereby the mismatched DNA molecule bound to the homolog dissociates therefrom. According to this variation, the method can be used as a method for purifying a mismatched DNA molecule from a population of DNA molecules which includes both mismatched and non-mismatched DNA molecules. By way of example, the population of DNA molecules may be made by annealing one or more single-stranded DNA molecules each having the consensus nucleotide sequence of a gene with single-stranded DNA molecules made by reverse transcription of mRNA obtained from an organism such as a mammal, whereby duplex DNA molecules not having a mismatched region are formed between single-stranded DNA molecules which are complementary to one another, and duplex DNA molecules having a mismatched region are formed between single-stranded DNA molecules which are complementary in some regions, but are not complementary in at least one region bounded by

complementary regions. Thus, the method of the invention of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules may be used to detect expression of a gene having a nucleotide sequence which differs from the consensus sequence of the gene in an organism.

The methods of the invention also include a method of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence. To perform this method, a first single-stranded DNA molecule having the sample nucleotide sequence is annealed with a second single-stranded DNA molecule having a nucleotide sequence which is completely complementary to the reference sequence to form a duplex DNA molecule. If there is a difference between the sample sequence and the reference sequence then the duplex DNA molecule has at least one mismatched region. According to the method, after forming the duplex DNA molecule, the duplex DNA molecule is contacted with a MutS homolog in the presence of a solution comprising an ADP molecule. Thus, if the duplex DNA molecule has a mismatched region, the homolog will bind to it. Next, according to the method, it is determined whether the homolog binds specifically to the duplex DNA molecule. Specific binding of the homolog to the duplex DNA molecule is an indication that there is a difference between the sample nucleotide sequence and the reference nucleotide sequence.

In the method of the invention of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence, any method of determining whether the homolog binds specifically to the duplex DNA molecule may be used. By way of example, the gel mobility shift assay or the DNase footprint assay described herein in Example 1 may be used to detect specific binding of the MutS homolog to the duplex DNA molecule. Also by way of example, after contacting the duplex DNA molecule with the MutS homolog, the duplex DNA molecule may be contacted with a membrane, such as a nitrocellulose membrane, under conditions such that double-stranded DNA does not bind to the membrane, but homolog-bound double-stranded DNA does bind to the membrane. Such conditions vary with the type of membrane used and are known in the art. Further by way of example, specific binding

of the homolog to the duplex DNA molecule may be detected by comparing the amount of the homolog bound to the duplex DNA molecule with the amount of the homolog bound to other duplex DNA molecules of similar size which are either known to contain mismatches or are known not to contain mismatches. Binding of a greater amount of the homolog to the duplex DNA molecule than the amount that binds to other duplex DNA molecules known not to contain mismatches is an indication that the duplex DNA molecule contains at least one mismatched region. Binding of a lesser amount of the homolog to the duplex DNA molecule than the amount that binds to other duplex DNA molecules known to contain mismatches is an indication that the duplex DNA molecule does not contain a mismatched region.

In a preferred method of detecting specific binding of the MutS homolog to the duplex DNA molecule, an optical affinity biosensor system (OABS) is used to detect specific binding. In an OABS system such as the IAsys™ system (Affinity Sensors, Cambridge, United Kingdom), binding and dissociation events can be detected as one molecule in solution binds to or dissociates from another molecule immobilized on a detector surface of the system. Thus, an OABS may be used to detect specific binding between the MutS homolog and the duplex DNA molecule having a mismatched region in any of the methods of the invention by immobilizing either the homolog or the DNA molecule on the detector surface of the OABS. Specific binding may be differentiated from non-specific binding by comparing binding of a MutS homolog to a duplex DNA molecule known to comprise a mismatched region and binding of the homolog to a duplex DNA molecule known not to comprise a mismatched region.

The sample nucleotide sequence and the reference nucleotide sequence in the just-described method may be any nucleotide sequence. In one embodiment of the method, the sample nucleotide sequences comprises the sequence of a region of a gene obtained from a first organism and the reference nucleotide sequence comprises the sequence of the region obtained from a second organism. By way of example, the first and the second organism may be members of the same species, such as *Homo*

sapiens. In another embodiment of the method, the sample nucleotide sequence comprises the sequence of a region of a gene obtained from an organism and the reference nucleotide sequence comprises a consensus nucleotide sequence of the region. By way of example, the gene may be the human *msh2* gene.

The invention also includes a kit for separating a mismatched duplex DNA molecule from a population of molecules. The kit comprises a MutS homolog, a linker for binding the MutS homolog to a solid support, and an ADP molecule. The kit is useful, for example, for performing the method of the invention of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules, as described herein. To use the kit according to this method, the MutS homolog is contacted with the population of duplex DNA molecules including at least one having a mismatched region, whereby the homolog binds to the mismatched region. The linker is used to bind the homolog to a solid support, either before or after contacting the homolog and the population. After the duplex DNA molecule having a mismatched region is bound to the homolog and the homolog is linked to the solid support, the duplex DNA molecule having a mismatched region is segregated from the population by virtue of being fixed, via the homolog, to the solid support. The solid support may be physically separated from the population if separation of the duplex DNA molecule having a mismatched region and the other DNA molecules of the population is desired. The kit may further comprise the solid support, such as polystyrene beads or a polystyrene microwell plate.

The methods of the invention include a method of detecting a predisposition of a mammal to carcinogenesis. This method is essentially the same as the method of the invention of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence, except that the sample nucleotide sequence is the nucleotide sequence of a region of a mammalian gene and the reference nucleotide sequence is the consensus nucleotide sequence of the region. The mammalian gene is selected from the group consisting of the *msh2* gene, the *msh3* gene, the *msh6* gene, the *mlh1* gene, the *pms2* gene, the *brca1* gene, the *brca2* gene, the

pten gene, and the *p53* gene. Detection of a difference between the sample nucleotide sequence and the reference nucleotide sequence is an indication that the mammal is predisposed to carcinogenesis. In a particularly contemplated embodiment, the mammalian gene is the human *msh2* gene the predisposition to carcinogenesis detected by the method is predisposition of a human for hereditary non-polyposis colorectal cancer.

The invention also includes a method of fractionating a population of DNA molecules. This method takes advantage of the ability of a MutS homolog to bind a duplex DNA molecule having a mismatched region in the presence of ADP, and the ability of the homolog to dissociate from the mismatched DNA molecule in the presence of ATP. The method of the invention of fractionating a population of DNA molecules comprises contacting the population with a MutS homolog, segregating the homolog from the population, and thereafter contacting the homolog with a solution comprising an ATP molecule. A mismatched duplex DNA molecule of the population binds to the homolog in the presence of ADP, is separated from the population when the homolog is segregated from the population, and is released from the homolog in the presence of ATP, whereby the population is fractionated. The population may be collected in one or more fractions after segregating the homolog therefrom, and the mismatched duplex DNA molecule may be collected in one or more additional fractions during or after contacting the homolog with ATP. By way of example, the homolog is fixed to a solid support, the population is contacted with the solid support by contacting the support with a suspension comprising the population, the solid support is rinsed with a solution which does not comprise the population, and then the solid support is rinsed with a solution comprising ATP. In one embodiment, a solution comprising ATP at a concentration that increases over time is provided continuously to the solid support, whereby a first mismatched duplex DNA molecule which is displaced from the homolog at a relatively low concentration of ATP can be collected in a first fraction and a second mismatched duplex DNA molecule which is displaced from the homolog at a relatively high concentration of ATP can be collected in a

second fraction. In another embodiment of this method, the solid support is a chromatography medium, such as agarose beads.

The methods of the invention further include a method of selectively amplifying at least one mismatched duplex DNA molecule in a population of duplex DNA molecules. To perform this method, a MutS homolog is contacted with the population in the presence of a solution comprising an ADP molecule, the homolog and a mismatched duplex DNA molecule bound thereto are segregated from the population, and the mismatched duplex DNA molecule is amplified using a PCR technique, whereby each of the at least one mismatched duplex DNA molecule is selectively amplified. The population of duplex DNA molecules may be any duplex DNA molecules, such as a population of duplex DNA molecules made by contacting a nucleic acid derived from an organism with at least one pair of PCR primers which can be used to amplify a region of a gene of the organism and performing PCR using the nucleic acid and the pair of PCR primers. The population may also, for example, be a cDNA library prepared using mRNA obtained from the organism. In one embodiment of this method, the method further comprises adding a first tail sequence to the 3'-end of the coding strand of the mismatched duplex DNA molecule and adding a second tail sequence to the 3'-end of the noncoding strand of the mismatched duplex DNA molecule prior to amplifying the mismatched duplex DNA molecule, and using two PCR primers, each of which is complementary to either the first or the second tail sequence. The sequences may be added, for example, by incorporating one of the first or the second tail sequences into one primer used to amplify a duplex DNA molecule and the other of the first or the second tail sequences into the other primer used to amplify the duplex DNA molecule. The tail sequences may also be added to the DNA molecule by blunt-end ligation of a hybrid DNA molecule comprising each of the first and the second tail sequences to the duplex DNA molecule, followed by enzymatic cleavage of the tail DNA molecule, whereby a portion of the tail DNA molecule comprising the first tail sequence remains attached to the 3'-end of one strand of the duplex DNA molecule and a portion of the tail DNA molecule comprising the second

tail sequence remains attached to the 3'-end of the other strand of the duplex DNA molecule. In another embodiment of this method, the method is repeated at least twice to improve the purity of the mismatched duplex DNA molecules that are amplified.

The invention also include a method of detecting the presence of a genetic polymorphism in a eukaryotic genome. A genetic polymorphism is a difference between the nucleotide sequence of the one copy of a gene in a chromosome and the nucleotide sequence of the other copy of the gene in the same chromosome. This method comprises amplifying a region of each of two copies of the desired gene to yield an amplified first copy and an amplified second copy. Then the first and the second copy are mixed together, denatured and are subsequently incubated under annealing conditions. The annealed mixture is contacted with a MutS homolog in the presence of a solution comprising an ADP molecule, the homolog is separated from the mixture, and the presence or absence of a nucleic acid specifically bound to the homolog is assessed. The presence of a nucleic acid comprising a DNA strand from the first copy and a DNA strand from the second copy and specifically bound to the homolog is an indication of the presence of a genetic polymorphism. Well known PCR technology can be used to amplify each copy of the gene. In one embodiment, the primers used in the PCR procedure for amplifying each copy are complementary to the intronic region immediately adjacent an exon of the gene. The mixture may be contacted with the MutS homolog using any of the methods described herein.

In the just-described method, detecting the presence or absence of a nucleic acid specifically bound to the MutS homolog can be performed using any known method of detecting the presence of a nucleic acid including, for example, detection of a shift in the ratio of the absorbance of the homolog at 280 nanometers to the absorbance of the homolog at 260 nanometers, whereby an decrease in this ratio is an indication that a nucleic acid is associated with the homolog. Further by way of example, the presence of the nucleic acid can be detected by detecting the presence of a radioactive, fluorescent, or biotinylated label incorporated into the nucleic acid using known methods. A nucleic acid comprising a DNA strand from the first copy of the

gene and a DNA strand from the second copy can be detected by sequencing both strands of the nucleic acid and comparing those sequences. If the two strands of the nucleic acid are from the same copy of the gene, then the two strands should be completely complementary. Thus, if the two strands are not completely complementary, then the two strands are derived from different copies of the gene. Identification of one or more regions of non-complementarity between the two strands is an indication of the presence of a genetic polymorphism. Gel mobility shift assays, DNase footprint assays, or OABS detection methods may also be used to detect the presence or absence of the nucleic acid specifically bound to the homolog.

In one embodiment of the method of the invention of detecting the presence of a genetic polymorphism, a nucleic acid specifically bound to the homolog is amplified prior to detecting the presence or absence of a nucleic acid associated with the homolog. This amplification may be accomplished, for example, by repeating the amplification procedure used in the method to amplify the region of the two copies of the desired gene, except that a nucleic acid specifically bound to the homolog is used in place of a nucleic acid derived from the genome of the animal. Amplification of this nucleic acid facilitates easier detection of the presence or absence of a nucleic acid specifically bound to the homolog because the amount of the nucleic acid is increased relative to the amount of a potentially contaminating nucleic acid not specifically bound to the homolog.

The invention also includes a composition for segregating mismatched duplex DNA molecules from a population of DNA molecules. The composition comprises a MutS homolog bound to a solid support, wherein the support is in liquid contact with a solution comprising an ADP molecule. The composition is used by contacting the population of DNA molecules with the composition and then segregating the composition from the population. Any of the methods described herein for segregating a MutS homolog bound to a solid support from a population of DNA molecules may be used to segregate the composition from the population. By contacting the composition with the population, a mismatched duplex DNA molecule

binds to the homolog of the composition. By segregating the composition from the population, the mismatched duplex DNA molecule bound to the composition is segregated from the population. The mismatched duplex DNA molecule may be dissociated from the composition by contacting the composition with a solution comprising an ATP molecule. The solid support for the composition may be any solid support to which a MutS homolog may be bound, such as polystyrene support linked to the homolog by an antibody which is bound to the support and which specifically binds to the homolog, for example. The solid support may have any form including, but not limited to a microwell plate, a porous membrane, a non-porous membrane, an insoluble particle, a chromatography medium, and a gel.

The invention also includes a kit for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence. The kit comprises a pair of primers adjacent to the region for amplifying the region, a duplex DNA molecule having the reference nucleotide sequence, a solid support, a MutS homolog, a linker for binding the homolog to the solid support, and an ADP molecule. The kit is used by amplifying the region using the pair of primers to yield an amplified DNA molecule, mixing the amplified DNA molecule and the duplex DNA molecule having the reference nucleotide sequence to form a mixture. The nucleic acids in the mixture are denatured and are annealed. The MutS homolog is added to the mixture and is bound to a solid support. The solid support is segregated from the mixture, and the presence or absence of a nucleic acid specifically bound to the homolog is assessed. The presence of a nucleic acid specifically bound to the homolog is an indication that the nucleotide sequence of the region differs from the reference nucleic acid sequence. Well known PCR methods may be used to amplify the region using the primers, and well known molecular biology methods may be used to denature and anneal the nucleic acids in the mixture. The mixture may be formed before or after denaturing the amplified DNA molecule and the duplex DNA molecule having the reference nucleotide sequence. The homolog may be bound to the solid support before or after contacting the mixture with the homolog. Any of the methods described herein for

detecting a nucleic acid specifically bound to the homolog may be used with the kit of the invention for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence.

In one embodiment of the kit of the invention for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence, the pair of primers comprises a sense primer having a first tail sequence and an antisense primer having a second tail sequence. In this embodiment, the genomic region is amplified using the pair of primers, and the amplified DNA molecule is mixed, denatured, and annealed with the duplex DNA molecule having the reference nucleotide sequence. The mixture is contacted with the homolog. The homolog is bound to the solid support and is then segregated from the population. Next, the homolog is contacted with a solution comprising ATP or a high salt concentration to cause a mismatched DNA duplex molecule specifically bound to the homolog to be dissociated therefrom. PCR primers complementary to the first tail sequence and the second tail sequence are used to amplify the mismatched DNA duplex molecule. This embodiment has the advantage that amplification of DNA molecules non-specifically bound to the homolog is minimized.

The invention also includes a nonhuman animal which is nullizygous for both the *Msh2* gene and the *p53* gene and methods of making and using the nonhuman animal, as described herein in Example 2.

The invention is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these examples but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example 1. The Human Mismatch Recognition Complex hMSH2/hMSH6 Functions as a Molecular Switch

Adenine nucleotide binding by the human mismatch recognition protein complex formed by association of the hMSH2 and hMSH6 proteins, is a novel

molecular switch. The hMSH2/hMSH6 protein complex is "ON" (i.e. it binds to mismatched DNA) in the ADP-bound form, and "OFF" (i.e. it dissociates from and does not bind to mismatched DNA) in the ATP-bound form. The data presented herein establish that the switch is 'turned OFF' by displacement of complex-bound ADP by ATP. ATP-bound complex is recycled to the ADP-bound form, which is capable of binding to mismatched DNA, by intrinsic ATPase activity of the complex.

The materials and methods used in the experiments presented in this Example are now described.

Overexpression and purification of hMSH2-hMSH6

Clones encoding hMSH2 and those encoding hMSH6 have been described (Acharya et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:13629-13634; Fishel et al., 1993, Cell 75:1027-1038). In the experiments described herein, the clone encoding hMSH6 was modified to further encode six histidine residues at the amino terminus of the hMSH6 protein molecule.

hMSH2 and hMSH6 were overexpressed in SF9 insect cells using the pFastBac™ dual expression vector (Gibco BRL, Grand Island, NY) as described in the Bac-to-Bac™ baculovirus expression systems protocol (Gibco BRL, Grand Island, NY). SF9 cells suspended in approximately 400 milliliters culture medium were infected using the vector, and were then cultured for 48 hours to achieve a cell density of approximately 10^6 SF9 cells per milliliter. The cells contained in 200 milliliter aliquots of SF9 cells were harvested by centrifugation at 200 × g, resuspended in 10 milliliters of buffer A, and frozen at -80°C. Buffer A comprised 300 millimolar NaCl, 20 millimolar imidizole, 25 millimolar HEPES buffer adjusted to pH 7.8 using NaOH, 10% (v/v) glycerol, 0.5 millimolar phenylmethylsulfonylfluoride (PMSF), 0.8 micrograms per milliliter pepstatin, and 0.8 micrograms per milliliter leupeptin.

Cell extracts were prepared by thawing the cells, passing the cells through a 25 gauge needle, and then ultracentrifuging the extract at 40,000 rotations per minute in a Beckman Ti60 rotor for 70 minutes, according to known methods. About 100 milliliters of infected cells yielded approximately 2 milligrams of hMSH2-hMSH6

protein complex. All of the following protein purification procedures in this Example were carried out at 4°C.

The supernatant was applied to a 2 milliliter nickel-NTA Superflow™ column (Qiagen, Chatsworth, CA) at a flow rate of 0.15 milliliters per minute using a Pharmacia FPLC system. The column was washed by passing 35 milliliters of buffer A through the column. After washing the column, the hMSH2/hMSH6 complex was eluted by applying 30 milliliters of buffer A comprising a linear gradient of imidizole to the column and collecting the eluent from the column in fractions, wherein the concentration of imidizole was varied from 20 millimolar to 200 millimolar. The hMSH2/hMSH6 complex eluted in fractions containing approximately 70 millimolar imidizole.

Fractions from the nickel-NTA column which contained peak amounts of the complex were loaded at a flow rate of 0.2 milliliters per minute directly onto a 1 milliliter PBE 94 column (a polybuffer exchange column obtained from Pharmacia, Upsala Sweden) which had been equilibrated with buffer B. Buffer B comprised 300 millimolar NaCl, 25 millimolar HEPES buffer adjusted to pH 7.8 using NaOH, 1 millimolar dithiothreitol (DTT), 0.1 millimolar ethylenediaminetetraacetic acid (EDTA), 10% (v/v) glycerol, 0.5 millimolar PMSF, 0.8 micrograms per milliliter pepstatin, and 0.8 micrograms per milliliter leupeptin. The PBE 94 column was washed by passing 10 milliliters of buffer B through the column. After washing the column, the hMSH2/hMSH6 complex was eluted by applying 20 milliliters of buffer B comprising a linear gradient of NaCl to the column and collecting the eluent from the column in fractions, wherein the concentration of NaCl was varied from 300 millimolar to 1 molar. The hMSH2/hMSH6 complex eluted from the PBE 94 column in fractions containing approximately 575 millimolar NaCl.

Fractions collected from the PBE 94 column which contained peak amounts of the complex were dialyzed twice for two hours against 2 liters of a solution comprising 100 millimolar NaCl, 25 millimolar HEPES buffer adjusted to pH 7.8 using NaOH, 1 millimolar DTT, 0.1 millimolar EDTA, and 20% (v/v) glycerol. Aliquots of

the dialyzed solution containing the complex were frozen using liquid nitrogen and stored at -80°C for several months without detectable loss of activity.

hMSH2, hMSH6, and bovine serum albumin (BSA) contain nearly identical percentages (12%, 14%, and 13%, respectively) of arginine and heterocyclic amino acids, the amino acids known to interact with the Coomassie Brilliant Blue stain. Protein concentration in an aliquot comprising the hMSH2-hMSH6 complex was determined by subjecting a portion of the aliquot to SDS-PAGE using a 6% (w/v) acrylamide gel, subjecting a known amount of BSA (Boehringer Mannheim, Indianapolis, IN) to SDS-PAGE using a 6% (w/v) acrylamide gel, staining the SDS-PAGE gels with Coomassie Brilliant Blue, and comparing the intensities of the protein bands in the gels to a BSA standard on a Coomassie stained 6% SDS PAGE to calculate protein concentration. The intensities of stained protein bands were measured using BioRad Gel Doc and Molecular Analyst™ software. This protein quantitation method revealed the hMSH2 and hMSH6 proteins to be in near exact equimolar proportion in the complex formed between the two proteins.

Preparation of 39- and 81-base pair oligonucleotide probes

The sequence of the 39-base pair oligonucleotide used in the experiments presented in this Example was: 5'-CGG CGA ATT CCA CCA AGC TTG ATC GCT CGA GGT ACC AGG-3' (SEQ ID NO:1). The homologous 39-base pair DNA substrate used in the experiments presented in this Example was made by annealing the 39-base pair oligonucleotide with an oligonucleotide (SEQ ID NO:2) which was completely complementary thereto. The G/T mismatched 39-base pair DNA substrate used in the experiments presented in this Example was made by annealing the 39-base pair oligonucleotide with an oligonucleotide (SEQ ID NO:3) which was completely complementary thereto, except that the oligonucleotide contained a G residue at the nucleotide position complementary to the T residue at position 20 (numbered in the direction extending from the 5' end to the 3' end) of the 39-base pair oligonucleotide. SEQ ID NO: 2 and SEQ ID NO: 3 are listed in Figure 10.

The nucleotide sequence of the 81-base pair oligonucleotide used in the experiments described in this Example was: 5'-AAA GCT GGA GCT GAA GCT TAG CTT AGG ATC ATC GAG GAT CGA GCT CGG TGC AAT TCA GCG GTA CCC AAT TCG CCC TAT AGT-3' (SEQ ID NO: 4). The homologous 81-base pair DNA substrate used in the experiments presented in this Example was made by annealing the 81-base pair oligonucleotide with an oligonucleotide (SEQ ID NO: 5) which was completely complementary thereto. The G/T mismatched 81-base pair DNA substrate used in the experiments presented in this Example was made by annealing the 81-base pair oligonucleotide with an oligonucleotide (having the nucleotide sequence listed in SEQ ID NO: 6) which was completely complementary thereto, except that the oligonucleotide contained a T residue at the nucleotide position complementary to the G residue at position 41 (numbered in the direction extending from the 5' end to the 3' end) of the 81-base pair oligonucleotide. SEQ ID NO: 5 and SEQ ID NO: 6 are listed in Figure 10.

^{32}P-end-labeled DNA substrates were prepared by incubating single stranded oligonucleotides in the presence of T4 polynucleotide kinase (Promega Corp., Madison, WI) and [^{32}P]γ-ATP (NEN Dupont, Wilmington, DE). Excess label was separated from the labeled DNA substrates using a Centrisep™ column (Princeton Separations, Princeton, NJ) per the manufacturer's instructions.

Labeled DNA substrate was annealed with a single-stranded DNA molecule which was either completely complementary thereto or contained a single G/T mismatch. To anneal the labeled DNA substrate with the single-stranded DNA molecule, the labeled molecule was suspended in a solution comprising a 10-fold excess of the single-stranded DNA molecule, 10 millimolar Tris buffer which had been adjusted to pH 7.5 using HCl, 100 millimolar NaCl, and 1 millimolar EDTA. The suspension was heated to 95°C and then slowly cooled to 55°C and was maintained at this temperature for twelve hours. Single-stranded DNA was removed from the suspension by incubating the suspension with benzoylated naphthoylated DEAE cellulose (BND cellulose, Sigma Chemical Co., St. Louis, MO) for twenty minutes in

the presence of a solution comprising 1.5 molar NaCl, 20 millimolar Tris buffer which had been adjusted to pH 7.5 using HCl, and 0.5 millimolar EDTA. BND cellulose was then pelleted by centrifuging the suspension for about five minutes using an Eppendorf bench-top centrifuge. Double-stranded DNA, which remained in the supernatant, was separated from the BND cellulose by filtration and was then precipitated by adding ethanol to the supernatant. The double-stranded labeled DNA substrate was resuspended in a solution comprising 10 millimolar Tris buffer which had been adjusted to pH 7.5 using HCl, 100 millimolar NaCl, and 1 millimolar EDTA. Single-stranded DNA could not be detected in the solution, as assessed by 4% (w/v) native PAGE separation of the nucleotides in the solution. Non-^{32}P-labeled oligonucleotides were prepared using analogous methods.

Gel mobility shift assays

Gel mobility shift assays were performed by incubating a complex comprising an hMSH2 protein molecule and an hMSH6 protein molecule and 9 femtomoles of either the ^{32}P-labeled homologous 81-base pair DNA substrate or the ^{32}P-labeled G/T-mismatched 81-base pair DNA substrate in a buffer comprising 50 millimolar NaCl, 25 millimolar HEPES buffer which had been adjusted to pH 7.5 using NaOH, 1 millimolar DTT, 0.01 millimolar EDTA, and 15% (v/v) glycerol. The buffer included 10 nanograms per microliter of poly dI-dC (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Poly dI-dC is an alternating nucleic acid polymer which does not interfere with binding of the hMSH2/hMSH6 complex to DNA. In certain experiments described herein, the incubation mixture further comprised selected concentrations of nucleotides or non-labeled DNA. In other experiments described herein, the incubation mixture further comprised 1 millimolar $MgCl_2$ or 5 millimolar EDTA. Except as otherwise described herein, each incubation mixture had a volume of 20 microliters and was incubated for fifteen minutes at 37°C and then immediately placed on ice. Each incubation mixture was applied to a gel comprising 4% (w/v) polyacrylamide (29:1 ratio of acrylamide:*bis*-acrylamide) 4% (v/v) glycerol, 40 millimolar Tris acetate buffer (pH 7.8), and 1 millimolar EDTA. Electrophoresis was

performed by applying 200 volts to the gel for two hours. Following electrophoresis, each gel was dried and quantitated using a phosphoimaging device obtained from Molecular Dynamics.

Footprint assays

Incubation of the hMSH2/hMSH6 complex with ^{32}P-labeled DNA substrates was performed as described for gel mobility shift assays, except that 18 femtomoles of ^{32}P-labeled DNA substrate was used in each assay. Following incubation, 80 microliters of a buffer comprising 50 millimolar NaCl, 25 millimolar HEPES buffer which had been adjusted to pH 7.8 using NaOH, 1 millimolar DTT, 10 nanograms per microliter poly dI-dC, 1.25 millimolar $CaCl_2$, 3.1 millimolar $MgCl_2$, 10% (v/v) glycerol, and 33 picograms per microliter DNase (Boehringer Mannheim, Indianapolis, IN) was added to each incubation mixture. The mixtures were incubated at 37°C for an additional three minutes, and then 0.7 milliliters of a solution having a pH of 5.2 and comprising 95% (v/v) ethanol and 180 millimolar sodium acetate was added to each mixture to halt the DNase reaction and to precipitate the nucleic acids present in the mixture.

DNase-treated nucleic acids were resuspended in 4 microliters of a solution comprising 80% (v/v) formamide, 10 millimolar NaOH, 1 millimolar EDTA, and 0.1 % (w/v) bromophenol blue. The suspension was heated at 90°C for five minutes and was applied to a gel comprising 8% (w/v) polyacrylamide (29:1 ratio of acrylamide:*bis*-acrylamide), 90 millimolar tris-borate buffer (pH 8), and 2 millimolar EDTA. Following electrophoresis for 2 hours at 200 volts, each gel was dried and imaged on a phosphoimaging device. Individual bases of the 81-base pair DNA substrates were identified by Maxam-Gilbert sequencing reactions performed as described (Ausubel et al., 1994, Current Protocols in Molecular Biology, 8th Ed., Janssen, ed., John Wiley & Sons, Inc., Boston).

ATPase assays

ATPase activity was measured in a reaction mixture comprising 20 microliters of Buffer P, 500 micromolar non-labeled ATP (except where indicated),

and 16.5 nanomolar [^{32}P]γ-ATP. Buffer P comprised 40 millimolar HEPES which had been adjusted to pH 7.8 using NaOH, 75 millimolar NaCl, 10 millimolar $MgCl_2$, 1.75 millimolar DTT, and 0.075 millimolar EDTA, and 15 % (v/v) glycerol. Steady state reaction measurements were made using 60 nanomolar hMSH2/hMSH6 complex and either 240 nanomolar homoduplex 39-base pair DNA substrate or 240 nanomolar G/T mismatched 39-base pair DNA substrate. Reaction mixtures were incubated at 37°C for thirty minutes, and the reaction was stopped by addition of 400 microliters of a solution comprising 10% (w/v) activated charcoal (Sigma Chemical Co., St. Louis, MO) and 1 millimolar EDTA. Charcoal was pelleted by centrifuging the mixture at 10,000 rotations per minute for ten minutes. The ^{32}P content of duplicate 100 microliter aliquots of the supernatant was assessed by liquid scintillation.

Initial velocity measurements were made by incubating the hMSH2/hMSH6 complex for ten minutes at 25°C in a reaction mixture comprising one volume Buffer P containing no $MgCl_2$, 200 nanomolar non-labeled ATP, and 16.5 nanomolar [^{32}P]γ-ATP. To start the reaction, an equal volume of buffer P comprising 20 millimolar $MgCl_2$ and 1 millimolar non-labeled ATP was mixed with the reaction mixture, which raised the $MgCl_2$ and ATP concentrations to 10 millimolar and 500 micromolar, respectively. Aliquots were removed at selected times and electrophoresed as described herein. A control aliquot was removed and prepared for electrophoresis prior to addition of the $MgCl_2$-containing Buffer P to the reaction mixture.

ADP exchange assays

The ADP-ATP exchange rate was determined in a reaction mixture which comprised Buffer Q, 2.3 micromolar [^{3}H]-ADP, and 60 nanomolar hMSH2/-hMSH6 complex. Buffer Q comprised 25 millimolar HEPES which had been adjusted to pH 7.8 using NaOH, 75 millimolar NaCl, 10 millimolar $MgCl_2$, 1 millimolar DTT, and 15% (v/v) glycerol. This reaction mixture was incubated for ten minutes at room temperature. 240 nanomolar G/T-mismatched 39-base pair DNA substrate was added to the reaction mixture, and the incubation was continued for an additional ten minutes.

The final volume of the reaction mixture was 10 microliters. The order of addition of DNA and ADP did not affect the kinetic results obtained using this assay. An equal volume Buffer Q comprising 1 millimolar non-labeled ATP was then added to the reaction mixture. Reactions were incubated at 25°C for a selected time and then halted by diluting the reaction mixture with 4 milliliters of an ice-cold stop buffer comprising 25 millimolar HEPES which had been adjusted to pH 7.8 using NaOH, 100 millimolar NaCl, and 10 millimolar $MgCl_2$.

Each halted reaction mixture was immediately filtered on a HAWP nitrocellulose membrane (Millipore, Bedford, MA) and washed thrice with 4 milliliters of the ice-cold stop buffer. Each filter was air dried and incubated overnight in a standard scintillation cocktail. Radioactivity retained on the filters was quantified using a Beckman scintillation counter. A control reaction mixture was prepared by not adding the Buffer Q comprising 1 millimolar non-labeled ATP to the reaction mixture. The amount of [^{3}H]-ADP retained on the membrane to which the control reaction mixture was applied was considered to correspond to the amount of radioactivity retained when 100% of the complex had [^{3}H]-ADP bound thereto.

Thin Layer Chromatography (TLC) Analysis

TLC was used to determine the composition of an ATPase reaction mixture which was prepared as described herein in the presence of the G/T-mismatched 39-base pair DNA substrate, 15 micromolar ATP, and 0.01 micromolar [^{32}P]α-ATP and which was permitted to react for twenty minutes at 37°C. TLC was performed as previously described (Fishel et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:36-40).

The results of the experiments presented in this Example are now described.

Overexpression and purification of the hMSH2-hMSH6 protein complex

hMSH2 and hMSH6 proteins were overexpressed in insect cells using a dual expression baculovirus vector, as indicated by the proteins found cell extract (Figure 1). Co-expression of hMSH2 and hMSH6 proteins resulted in formation of a completely soluble protein complex. Independent expression of either protein alone

resulted in formation of a substantial amount of insoluble protein product. hMSH2 and hMSH6 likely exist together as a highly stable complex *in vivo*, as judged by the results obtained in the Experiments described in this Example, the ability of investigators to copurify these two proteins from human cells (Drummond et al., 1995, Science 268:1909-1912), and the ability of these two proteins to interact *in vitro* (Acharya et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:13629-13634).

Purification of hMSH2 and hMSH6 from insect cells indicated that a stable heterodimer of the two proteins had been formed. Quantitative densitometry of Coomassie-stained products consistently revealed that the hMSH2 and hMSH6 subunits were present in an equimolar ratio, as was observed with the yeast MSH2/MSH6 protein complex (Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447). The purification methodology described herein yielded a protein preparation which was more than 95% homogeneous, which exhibited high MSH2/MSH6 activity, and which appeared to be free of any contaminating nucleic acid or nucleotide.

G/T mismatch binding by hMSH2-hMSH6 is a model for mismatch recognition

The hMSH2-hMSH6 protein complex has been demonstrated herein and by others to bind to the eight possible mismatched nucleotide combinations, as well as to a subset of single nucleotide insertion/deletion mismatches (Acharya et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:13629-13634; Drummond et al., 1995, Science 268:1909-1912; Hughes et al., 1992, J. Biol. Chem. 267:23876-23882). The G/T mismatch was chosen as a model for quantitative analysis of hMSH2-hMSH6 mismatch binding because of its apparently intermediate-to-high recognition specificity, as indicated, for example, by the data presented in Figures 2A-2D.

The apparent dissociation constant (K_d) was determined in a simple buffer system comprising neither an adenine nucleotide nor magnesium using the homologous 81-base pair DNA substrate and the G/T-mismatched 81-base pair DNA substrate described herein. Results obtained using both gel shift assays, as depicted in Figure 2A, and DNase footprint assays, as depicted in Figure 2C, indicated that K_d of

the hMSH2/hMSH6 complex for G/T mismatches was 20 ± 5 nanomolar. Binding of non-mismatched DNA to the complex was not saturable, even at homoduplex concentrations greater than 400 nanomolar.

The binding of the hMSH2/hMSH6 complex to a G/T mismatch is at least ten times more efficient than binding of hMSH2 alone to the G/T mismatch (Fishel et al., 1994, Science 266:1403-1405; Fishel et al., 1994, Cancer Res. 54:5539-5542; Mello et al., 1996, Chemistry & Biology 3:579-589). This observation indicates that formation of the protein complex enhances both the affinity and the specificity of hMSH2-binding to mismatched DNA (Acharya et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:13629-13634).

Gel mobility shift assays performed using the G/T-mismatched 39-base pair DNA substrate described herein or using the G/T-mismatched 81-base pair DNA substrate and a buffer comprising 2 millimolar $MgCl_2$ yielded results similar to those shown in Figure 2A. The hMSH2-hMSH6 complex appears to bind G/T mismatched DNA in multiple forms which are differentiable by gel mobility shift assay.

DNase footprint analysis of hMSH2/hMSH6 complex binding to the G/T-mismatched 81-base pair DNA substrate indicated that the complex asymmetrically protects about 25 nucleotides on both strands of the substrate. As shown in Figure 2C, there appeared to be two domains protected by the complex from cleavage by DNase. One domain appeared to be centered on the G/T mismatch in the substrate. The other domain was adjacent the domain centered on the G/T mismatch and was separated from that domain by a single DNase-sensitive nucleotide. These data are qualitatively similar to those observed in similar experiments using the *E. coli* and *T. aquaticus* MutS proteins (Su et al., 1986, Proc. Natl. Acad. Sci., U.S.A. 83:5057-5061; Su et al., 1988, J. Biol. Chem. 263:6829-6835; Biswas et al., 1997, J. Biol. Chem. 272: 13355-13364).

Although a shifted complex could be detected by gel mobility shift assay using homoduplex DNA, no specific DNase footprint could be identified, as indicated by the data presented in Figure 2D. Lack of saturatability and lack of a

specific footprint are consistent with the ability of the hMSH2/hMSH6 complex to weakly and non-specifically associate with homoduplex DNA.

Shifted complexes formed between the complex and homoduplex DNA and those formed between the complex and G/T-mismatched DNA migrated differently in gel mobility shift assays, as shown in Figures 2A and 2B. Homoduplex DNA-bound complex (designated 'NS' for 'non-specific' in Figure 2B) migrated more slowly than G/T-mismatched DNA-bound complex (designated 'S' for 'specific' in Figure 2A). These results suggest that homoduplex DNA-bound complex adopts a different conformation than mismatched DNA-bound complex. Alternatively, there may have been a greater quantity of the complex bound to homoduplex DNA than to mismatched DNA.

When the homoduplex 39-base pair DNA substrate described herein was contacted with the complex, no NS product was observed in the gel mobility shift assay. The DNA length dependence of NS product formation may result if a minimum number of base pairs were necessary to assume an alternative DNA and/or hMSH2- or hMSH6-protein conformation or to bind multiple hMSH2/hMSH6 molecules.

These results demonstrate the high specificity of complex binding to the G/T-mismatched 81-base pair DNA substrate. The binding was found to be quantitatively similar by both gel mobility shift and footprint analysis. In addition, a low level non-specific binding to duplex DNA was observed and found to be easily distinguished via its altered mobility using gel mobility shift analysis.

The hMSH2-hMSH6 complex converts ATP to ADP in the presence of mismatched DNA

Both bacterial and yeast MutS homologs have been shown to possess intrinsic low-level ATPase activity (Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447; Chi et al., 1994, J. Biol. Chem. 269: 29993-29997; Chi et al., 1994, J. Biol. Chem. 269:29984-29992; Habe et al., 1988, J. Bacteriol. 170:197-202). There are conflicting reports regarding the capacity of mismatched heteroduplex and/or homoduplex DNA to stimulate this intrinsic ATPase activity (Alani et al., 1997, Mol. Cell Biol. 17: 2436-

2447; Chi et al., 1994, J. Biol. Chem. 269: 29993-29997; Chi et al., 1994, J. Biol. Chem. 269:29984-29992).

It has been demonstrated herein that the hMSH2/hMSH6 complex possesses intrinsic DNA-dependent ATPase activity that is dependent upon the presence of magnesium as a cofactor, as indicated by the results illustrated in Figure 3A. Saturation of the ATPase activity by hMSH2-hMSH6 at protein concentrations above 0.6 micromolar are the likely result of limiting DNA, which was introduced at a fixed concentration of 240 nanomolar.

As indicated by the results presented in Figure 3B, thin layer chromatography revealed that the hMSH2-hMSH6 ATPase uniformly converts ATP to ADP and inorganic phosphate. Using Lineweaver-Burk analysis and Eadie-Hofstee analysis, it was determined that hMSH2/hMSH6 complex ATPase is most active in the presence of a G/T mismatch (Figure 3C). The value of k_{cat} using ATP and G/T-mismatched DNA as substrates was about 26 minute^{-1}. The value of K_m using ATP and G/T-mismatched DNA as substrates was about 46 micromolar. hMSH2/hMSH6 complex ATPase is substantially less active in the presence of homoduplex DNA. The value of k_{cat} using ATP and G/C-mismatched DNA as substrates was about 7.4 minute^{-1}. The value of K_m using ATP and G/C-mismatched DNA as substrates was about 23 micromolar. hMSH2/hMSH6 complex ATPase is substantially inactive in the absence of DNA. The value of k_{cat} using ATP alone as a substrate was about 0.9 minute^{-1}. The value of K_m using ATP alone as a substrate was about 10 micromolar.

ATPase activity stimulation was the same regardless of whether the homoduplex DNA had a length of 39 base pairs, 81 base pairs or 2,900 base pairs, and was also the same regardless of whether the mismatched DNA had a length of 39 base pairs or 81 base pairs. These results indicated that hMSH2/hMSH6 complex ATPase activity is not dependent upon DNA length.

It was observed that k_{cat} using ATP alone as a substrate was lower than k_{cat} using ATP and homoduplex DNA as a substrate and this value was lower than k_{cat} using ATP and mismatched DNA as substrates. However, K_m for ATP in the absence

of DNA was lower than K_m for ATP in the presence of homoduplex DNA, and this value was lower than K_m for ATP in the presence of mismatched DNA. These observations indicated that although the rate of hydrolysis is increased in the presence of a mismatch, the affinity for ATP is decreased. These results are qualitatively similar to the phenomenon of uncompetitive inhibition which may be ascribed to the presence of independent and separate binding sites as well as a ping-pong binding mechanism (Dixon et al., 1979, Enzymes, 3rd Ed., Academic Press, New York). Single-stranded DNA (ssDNA) was found to be the most potent stimulator of hMSH2/hMSH6 ATPase activity. Thus, the conflicting reports in the prior art regarding ATPase activities of related MutS homologues may have resulted from contamination by ssDNA leached from columns used to purify the homologues and/or non-annealed ssDNA that remained following preparation of oligonucleotide substrates.

hMSH2/hMSH6 mismatch binding is abolished in the presence of ATP in the absence of hydrolysis of ATP

Both bacterial and eukaryotic MutS homologs fail to form a specific complex with a mismatched oligonucleotide in the presence of ATP (Drummond et al., 1995, Science 268:1909-1912; Haber et al., 1991, EMBO. J. 10:2707-2715; Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447; Grilley et al., 1989, J. Biol. Chem. 264:1000-1004). Before the present invention, it was believed that ATP hydrolysis catalyzed by MutS protein drove translocation of the protein along a duplex DNA strand, causing dissociation of the protein from any mismatch with which it might be associated (Grilley et al., 1989, J. Biol. Chem. 264:1000-1004; Modrich, 1989, J. Biol. Chem. 264:6597-6600; Modrich, 1991, Annu. Rev. Genet. 25:229-253; Modrich et al., 1996, Annu. Rev. Biochem. 65:101-133; Allen et al., 1997, EMBO Journal 16:4467-4476). The suggestion that ATP hydrolysis was required for the mismatch release was based on the observation by others that adenylyl-imidodiphosphate (AMP-PNP), a non-hydrolyzable analog of ATP, does not alter mismatch binding (Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447; Drummond et al., 1995, Science 268:1909-1912).

The experiments described in this Example establish that the hMSH2/hMSH6 complex is released from a G/T-mismatched DNA substrate in the presence of ATP (Figures 4A and 4D. The value of IC_{50} (the concentration of ATP required to cause release of 50% of a population of complexes from a G/T-mismatched DNA substrate) was determined to be approximately 3 micromolar. Adenosine-5'-O-3-thiotriphosphate (ATP-γ-S), a poorly-hydrolyzable ATP analog (Sekimizu et al., 1987, Cell 50:259-265; Yu et al., 1992, J. Mol. Biol. 225:193-216), caused a similar release of the hMSH2/hMSH6 complex from a G/T-mismatched DNA substrate, the value of IC_{50} for ATP-γ-S being 3 micromolar (Figures 4B and 4D). Addition of ADP to the mismatch binding reaction mixture resulted increased binding affinity of the complex for the G/T-mismatched DNA substrate (Figures 4C and 4D.

The results presented in this Example demonstrate that release of the hMSH2/hMSH6 complex from a G/T-mismatched DNA substrate to which it is bound is not associated with ATP hydrolysis. This conclusion follows from the observations that release of the complex occurs in the absence of exogenous magnesium and that release of the complex from the substrate is effected by the presence of ATP-γ-S regardless of the presence or absence of magnesium. The presence of magnesium is absolutely required for hMSH2-hMSH6 dependent ATP hydrolysis. Furthermore, NS binding of hMSH2 to homoduplex DNA is insensitive to the addition of exogenous ATP. Thus, the presence of ATP affects only the ability of the hMSH2/hMSH6 protein complex to bind to mismatched DNA substrates. Binding of the complex to homoduplex DNA is not affected by ATP.

The presence of 2'-deoxy adenosine triphosphate (dATP) to the mismatch binding reaction mixture caused release of a G/T-mismatched DNA substrate from the hMSH2/hMSH6 protein complex, similarly to the release caused by the presence of ATP or ATP-γ-S in the mixture, as illustrated in Figure 5. No other nucleotide was found to stimulate the release of the G/T-mismatched DNA substrate from the complex.

Neither of two other non-hydrolyzable analogs of ATP, namely AMP-PNP and adenyl(β, γ-methylene)diphosphonate (AMP-PCP), caused release of the complex from the substrate. Equilibrium competition between each of these two analogs and ATP suggested that they bind to the complex and caused effects similar to those caused by ADP. Failure of AMP-PNP and AMP-PCP to stimulate release of mismatched DNA from the complex demonstrated that the interaction between the β-γ bridging oxygen atom of ATP and either the complex or the mismatched DNA substrate bound to the complex are for release of the substrate from the complex. Enzyme-nucleotide triphosphate complexes in which the β, γ oxygen atom interacts with either the enzyme or its substrate are not unknown. For example, the Ras GTPase binds GTP, and donation of a hydrogen bond to the β-γ bridging oxygen of GTP is thought to contribute to catalysis by the enzyme (Maegley et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:8160-8166).

The results presented in this example demonstrate that the hMSH2/hMSH6 complex binds to a mismatched DNA substrate in the presence of ADP, and that the substrate is released from the complex in the presence of ATP or dATP. Because ATP-induced release of the substrate from the complex does not require magnesium and is similarly induced by ATP-γ-S, ATP hydrolysis is not implicated in substrate release. As increasing amounts of ATP or ATP-γ-S were added to the mismatch binding reaction mixture, approximately 15% of S-shifted material gradually became reassociated with the DNA in the form of a NS-shifted complex (Figures 4A and 4B). This fraction was consistent with the amount of NS binding observed for homoduplex DNA at this concentration of the complex (Figure 2B). These results indicated that hMSH2/hMSH6 complex molecules which dissociated from mismatched substrate could reassociate with either the duplex arms or the ends of the substrate.

ATP hydrolysis catalyzed by the hMSH2/hMSH6 complex results in recovery of mismatch binding activity of the complex

To determine the role of ATP hydrolysis in mismatch recognition, ATP or ATP-γ-S was introduced into a mismatch binding reaction mixture in the absence of magnesium. As illustrated in Figures 4A, 4B, 4D, and 5, introduction of either compound resulted in release of the hMSH2/hMSH6 complex from the mismatched DNA substrate in the absence of hydrolysis of the compound. In experiments presented in Figures 6A, 6B, and 6C, magnesium was added to each reaction mixture, which was maintained at 37°C, and the G/T mismatch binding activity of hMSH2-hMSH6 was followed over time, with time zero corresponding to the time at which magnesium was added. In the reaction mixture comprising ATP, mismatched DNA substrate binding activity of the complex was initially low, nearly 70% of this activity was recovered after ten minutes of incubation at 37°C, and more than 95% of the activity was recovered fifty minutes after magnesium addition. Substantially less (about 22%) of mismatched DNA substrate binding activity was recovered in the reaction mixture to which ATP-γ-S was added. These results demonstrated that efficient hydrolysis by the complex is essential for recovery of the complex's mismatch binding activity. Substitution of ATP with dATP produced quantitatively similar recovery of mismatch binding activity (i.e. >95% recovery) following incubation at 37°C. Taken together, these results demonstrated that the intrinsic ATPase activity associated with the human hMSH2/hMSH6 complex is required for recovery from mismatch-release induced by binding to and/or exchange with, ATP or dATP..

Complete recovery of mismatched DNA substrate binding activity of the hMSH2/hMSH6 complex, which activity was abolished by exposing the complex to ATP, was achieved by increasing the ratio of the concentration of ADP to the ratio of ATP in the solution in which the complex was suspended (Figures 6D and 6E). In this competition experiment, mismatch binding reaction mixtures comprised 0.2 millimolar ATP, 1 millimolar $MgCl_2$, and a selected concentration of ADP from 0 to 3.2 millimolar. It was determined that a 2- to 3-fold excess of ADP to ATP resulted in reversal of approximately half of the release of substrate by the complex caused by the presence of ATP. Approximately complete reversal of substrate release caused by the

presence of ATP was achieved by providing a 16-fold excess of ADP to the mixture. A qualitatively similar, though functionally opposite, result was obtained when the competition was performed by including a fixed concentration of ADP in the reaction mixture and adding various concentrations of ATP. Thus, ADP and ATP are nearly equivalent in their ability to associate with the hMSH2/hMSH6 complex, but the two nucleotides elicited opposite functional effects on mismatch binding. ATP caused release of substrate bound to the complex, and ADP induced binding of the substrate to the complex. Therefore, ADP is responsible for mismatch binding recovery.

Taken together, these observations support the conclusion that the hMSH2/hMSH6 complex functions as a molecular switch, wherein the ATP- (or dATP-) bound complex is "OFF" (i.e. unable to bind a mismatched DNA substrate) and the ADP-bound complex is "ON" (i.e. able to bind a mismatched DNA substrate). A model of the role of the hMSH2/hMSH6 complex is illustrated in Figure 9.

ATP hydrolysis and ADP-ATP exchange determine mismatch binding functions of the hMSH2/hMSH6 complex

Steady-state analysis of an enzyme having ATPase activity reflects the rate-limiting step of the reaction, which can be either γ-phosphate hydrolysis or adenine nucleotide exchange, as indicated in Figure 7A. To understand the mechanism of the ATPase activity exhibited by the hMSH2/hMSH6 protein complex and to further define the rate-limiting steps, both hydrolysis and nucleotide exchange steps were directly examined.

Initial rate (i.e. single-turnover) analysis of an enzyme which exhibits ATPase activity involves direct examination of the rate of γ-phosphate hydrolysis, and was performed using a method which is similar to that used for the examination of regulators of G-protein signaling (RGS; Dohlman et al., 1997, J. Biol. Chem. 272:3871-3874). In these experiments, 0.2 micromolar [^{32}P]γ-ATP was contacted with hMSH2/hMSH6 protein complex in the absence of magnesium, yielding a complex having a [^{32}P]γ-ATP molecule bound thereto. At a selected time, magnesium and an excess of non-labeled ATP were added to the reaction mixture, and the rate of a single-

round of γ-phosphate hydrolysis was assessed. Subsequent rounds of hydrolysis were undetectable because the ATP hydrolyzed during those rounds was not labeled. Because the calculated K_{cat} for ATP at 37°C was in excess of 20 minute^{-1}, and because this rate was above the limit of detection of this methodology, these initial rate experiments were performed at 20°C. It was determined that the hMSH2/hMSH6 complex rapidly hydrolyzed ATP in either the presence or the absence of DNA. These results indicated that γ-phosphate hydrolysis was not the rate limiting step in the steady-state ATP hydrolysis by the complex.

The extent of ATP hydrolysis which could be detected was equivalent to the total number of hMSH2/hMSH6 complex molecules which could be bound to ^{32}P-labeled ATP prior to the addition of magnesium. The maximal extent of detectable ATP hydrolysis was determined to depend on the amount of the G/T-mismatched DNA substrate present in the reaction mixture during binding of labeled ATP to the complex (Figures 7B and 7C). When the concentration of the G/T-mismatched DNA substrate in to the reaction mixture exceeded the apparent K_d for G/T-mismatched DNA substrate (i.e. about 20 nanomolar), the maximal extent of ATP hydrolysis decreased (Figure 7C). This observation indicated that binding of the hMSH2/hMSH6 protein complex to a mismatched DNA molecule prior to binding of ATP to the complex inhibits binding of ATP to the mismatched DNA-bound complex. This observation is consistent with the pseudo-uncompetitive behavior deduced in the steady-state ATPase activity experiments described herein (Dixon et al., 1979, Enzymes, 3rd Ed., Academic Press, New York).

Adenine nucleotide exchange was assessed using a method similar to that used for guanine nucleotide exchange experiments involving G proteins. In these studies, [^{3}H]-ADP was contacted with hMSH2/hMSH6 protein complex in the presence of magnesium, yielding [^{3}H]-ADP-bound complex. At a selected time, an excess of non-labeled ATP was added to the reaction mixture, and the amount of ADP that remained bound to the complex was assessed at selected times.

In the absence of DNA, incomplete ADP nucleotide exchange was observed during a 15 minute reaction period. The half-life of the ADP-bound complex was greater than eight hundred seconds (Figure 7D. These results clearly suggest that in the absence of DNA, replacement of ADP by ATP is the rate limiting step for the hMSH2/hMSH6 complex ATPase activity.

In the presence of G/T-mismatched DNA substrate, nucleotide exchange was significantly more rapid, the half-life of the ADP-bound complex being less than two seconds, as indicated by the data depicted in Figure 7D. Thus, it was demonstrated that binding of the complex to a G/T-mismatched DNA substrate stimulated replacement of the labeled ADP molecule originally bound to the complex by a non-labeled ATP molecule.

Taken together with the results obtained from the single turnover hydrolysis experiments described herein, these observations indicated that in the absence of mismatched DNA, the hMSH2/hMSH6 protein complex is capable of a single ATP hydrolysis reaction that yields an ADP-bound complex. While in the ADP-bound form, the complex does not exchange ADP for ATP until the complex binds to a DNA mismatch. By binding to a mismatch, the ADP-bound complex becomes competent to exchange ADP for ATP. Exchange of ADP for ATP causes release of the complex from the mismatch. ATP-bound complex, no longer bound to mismatched DNA, is capable of catalyzing ATP hydrolysis, yielding ADP-bound complex, which is competent to bind to a DNA mismatch. These results indicate that the hMSH2/hMSH6 protein complex is a molecular switch controlled by the phosphorylation state of the adenine nucleotide bound thereto.

Release of the hMSH2/hMSH6 protein complex from a G/T-mismatched DNA substrate occurs by simple dissociation

Prior art models of mismatch recognition by MutS homologs implicated ATP-dependent translocation and/or treadmilling along DNA as a mechanism for association and dissociation of the homolog with a DNA mismatch (Modrich, 1989, J. Biol. Chem. 264:6597-6600; Modrich, 1991, Annu. Rev. Genet. 25:229-253; Modrich

et al., 1996, Annu. Rev. Biochem. 65:101-133; Allen et al., 1997, EMBO Journal 16:4467-4476). Common to all of these prior art models is a postulated time-dependent unidimensional homolog displacement mechanism which occurs whether the homolog is bound to duplex DNA or mismatched DNA. In contrast, a simple dissociation mechanism would exhibit rapid and two-dimensional displacement of the homolog from duplex DNA or mismatched DNA.

The ability to distinguish NS and S electrophoretic bands corresponding to the homologous 81-base pair DNA substrate-bound complex and the G/T-mismatched 81-base pair DNA substrate-bound complex (Figure 4A) provided an opportunity to examine the dissociation mechanism of the hMSH2/hMSH6 protein complex from the G/T-mismatched DNA substrate, as well as from homoduplex DNA. In these experiments, the G/T-mismatched DNA substrate was bound to hMSH2/hMSH6 substrate, and an excess of an unlabeled competitor DNA or an excess of ATP, or both, was added to the mixture. If a tracking or sliding mechanism of the prior art were operable for hMSH2/hMSH6 complex dissociation, it would be expected that a time-dependent loss of the S shifted electrophoretic band of G/T-mismatched DNA substrate-bound complex would be observed, and that a coincident gain of the NS electrophoretic band would be observed. If a simple dissociation mechanism were operable for hMSH2/hMSH6 complex dissociation, it would be expected that loss of the S shifted band would be observed without any coincident increase in the intensity of the NS shifted band because the vast excess of unlabeled homoduplex DNA would preclude secondary reassociation of the complex with the arms or ends of the labeled G/T-mismatched DNA substrate.

Three experiments were performed to determine the mechanism of hMSH2/hMSH6 protein complex dissociation from a labeled 81-base pair G/T-mismatched DNA substrate. The results of these experiments are illustrated in Figure 8.

In the first experiment, the stability of G/T-mismatched DNA substrate-bound hMSH2/hMSH6 complex was assessed by exposing the mismatched substrate-

bound complex to a 400-fold excess of non-labeled homoduplex DNA and observing the intensities of S shifted and NS shifted electrophoretic bands at selected times (Figure 8C). Examination of the gel depicted in Figure 8C indicated that the S-shifted electrophoretic band, and thus the amount of the G/T-mismatched DNA substrate-bound hMSH2/hMSH6 complex in the reaction mixture, was not reduced significantly over the ten minute incubation period. Thus, the half-life of the G/T-mismatched DNA substrate-bound hMSH2/hMSH6 complex was much greater than ten minutes, meaning that the mismatched substrate-bound complex is stable in the presence of a vast excess of homoduplex DNA.

In the second experiment, the stability of G/T-mismatched DNA substrate-bound hMSH2/hMSH6 complex was assessed by exposing the mismatched substrate-bound complex to ATP and observing the intensities of S shifted and NS shifted electrophoretic bands at selected times (Figure 8A). A gradual decrease in the intensity of the S shifted electrophoretic band was observed, the band having a half life of about twenty seconds. Concurrently with the decrease in the intensity of the S shifted electrophoretic band, a gradual but not quantitative increase in the intensity of the NS-shifted electrophoretic band was observed. This observation indicated that ATP induced a time-dependent reduction of specific binding of the hMSH2/hMSH6 complex to the mismatched DNA substrate and that at least a portion of the complex reassociated with the mismatched DNA substrate in a non-specific manner. However, this experiment did not distinguish between the tracking/sliding or simple dissociation and reassociation mechanisms.

In order to distinguish between tracking/sliding and simple dissociation and reassociation, a third experiment was performed. In this experiment, the stability of G/T-mismatched DNA substrate-bound hMSH2/hMSH6 complex was assessed by exposing the mismatched substrate-bound complex to both ATP and a 400-fold excess of non-labeled homoduplex DNA and observing the intensities of S shifted and NS shifted electrophoretic bands at selected times (Figure 8B). As in the second experiment, a gradual decrease in the intensity of the S shifted electrophoretic band was

observed, the half-life of the band again being about twenty seconds. This observation was consistent with ATP induction of dissociation of the complex from the mismatched DNA substrate. However, under these conditions, no increase in the intensity of the NS electrophoretic band was observed. Together, these observations indicate that in the presence of excess non-labeled homoduplex DNA, the dissociation of the complex from mismatched DNA does not proceed through the product corresponding to the NS electrophoretic band, but instead is instantaneous and irreversible. When excess non-labeled homoduplex DNA was added to the homologous 81-base pair DNA substrate, the NS electrophoretic band associated with the product formed by contacting the complex with DNA substrate (as in Figure 2B) could be detected (Figure 8D). This observation indicated that, even at 4°C, the product corresponding to the NS band was exceedingly unstable and that level of hMSH2/hMSH6 which remained associated with the DNA substrate was less than the lower limit of accurate quantitation using gel shift analysis.

Without intending to be bound to any particular theory or mechanism, it was concluded that a mechanism involving translocation or treadmilling by the hMSH2/hMSH6 protein complex is an unlikely explanation for the association and dissociation of the complex and mismatched DNA for three primary reasons. First, no intermediate (i.e. no NS shifted electrophoretic band) was observed during ATP-induced dissociation of the complex from the G/T-mismatched DNA substrate in the presence of an excess of unlabeled homologous DNA. Second, dissociation of the hMSH2/hMSH6 complex was too rapid to measure, even at low temperatures. Third, ATP hydrolysis was not required for any of the dissociation processes, as evidenced by the fact that these three experiments were performed in the absence of magnesium. Qualitatively identical results were obtained in the presence of magnesium. Together, the results of these three experiments are consistent with the idea that the release of the hMSH2/hMSH6 protein complex from a mismatched DNA occurs rapidly by a process of simple dissociation.

It is noted that a translocation mechanism involving binding of ATP followed by rapid unidimensional translocation of the complex a distance of at least 40 nucleotides in the absence of hydrolysis cannot be eliminated as a possible mechanism for the association and dissociation of the complex with mismatched DNA. Although this type of mechanism is unlikely, it is possible to visualize a process in which a step-translocation is followed by ATP hydrolysis, displacement of ADP by ATP, and another step-translocation. However, it is worth noting that steady-state ATPase activity of the hMSH2/hMSH6 protein complex is diminished in the presence of duplex DNA, relative to the steady-state ATPase activity of the complex in the presence of mismatched DNA. It is difficult to reconcile the opposing activities of an ATP-bound step-translocation away from the mismatch followed by an ADP-bound mismatch binding stage, into a simple model for mismatch repair.

The hMSH2/hMSH6 protein complex acts as a molecular switch in mismatch recognition

The discovery that the hMSH2/hMSH6 complex is a novel molecular switch which is activatable by ADP was made by reconciling numerous observations described herein. These observations are summarized as follows. ADP and ATP have opposing effects on the role of the hMSH2/hMSH6 complex in mismatched DNA binding. Release of mismatched DNA by the hMSH2/hMSH6 complex is not dependent upon ATP hydrolysis. Hydrolysis of ATP by the hMSH2/hMSH6 complex results in recovery of the mismatch binding ability of the complex. γ-Phosphate hydrolysis is not the rate limiting step of ATPase activity catalyzed by the hMSH2/hMSH6 complex. Displacement of ADP by ATP is the rate limiting step of ATPase activity catalyzed by the hMSH2/hMSH6 complex. Displacement of ADP from the hMSH2/hMSH6 complex by ATP is accelerated in the presence of mismatched DNA, but hydrolysis of the γ-phosphate bond is not accelerated. ATP-dependent release of mismatched DNA from the hMSH2/hMSH6 complex occurs rapidly and by simple dissociation. These observations indicate that γ-phosphate hydrolysis and displacement of ADP by ATP determine whether the hMSH2/hMSH6

complex binds to or is released from mismatched DNA (Figure 9). Recognition of the hMSH2/hMSH6 complex as a molecular switch supports the conclusion that it is a trigger for determining the timing of subsequent excision repair-related events.

Implications for mismatch repair

The number of hMSH2/hMSH6 complex molecules in the nucleus of a proliferating cell has been estimated to exceed one thousand (Drummond et al., 1995, Science 268:1909-1912; Wilson et al., 1995, Cancer Research 55:5146-5150; Meyers et al., 1997, Cancer Res. 57:206-208). The calculated K_d of about 20 nanomolar for mismatched DNA implies that a single mismatched nucleotide in a human cell is likely to be efficiently recognized and bound with high affinity by the hMSH2/hMSH6 complex. In the presence of ADP, this high affinity binding is nearly irreversible. Thus, dissociating the complex from the mismatched DNA in order to allow a subsequent excision repair event to proceed may be more difficult than binding the complex to the mismatch.

Without wishing to be bound by any particular theory or mechanism of operation, it is proposed that the tight binding of the complex to mismatched DNA acts as a flag for the assembly or nearby localization of cellular excision repair proteins or other factors. When the complete excision repair machinery is assembled nearby, displacement of ADP by ATP could be triggered by a component of the machinery, causing dissociation of the complex from the mismatched region. Exonuclease excision and replacement of the mismatched region may then proceed.

Once released from the mismatched nucleotides, the intrinsic ATPase activity of the hMSH2/hMSH6 complex could cause hydrolysis of the ATP molecule which displaced ADP, yielding the ADP-bound form of the complex that is competent for mismatch binding. As a free protein complex, hMSH2/hMSH6 does not efficiently exchange ADP bound thereto, providing a long-term mismatch recognition-competent molecule.

The enhanced ability ATP to displace ADP bound to the hMSH2/hMSH6 complex in the presence of mismatched DNA may be explained as

follows. It is possible that the hMSH2/hMSH6 complex oscillates between a mismatch recognition-competent (i.e. ADP-bound) form and a mismatch-recognition-incompetent (i.e. ATP-bound) form. Such oscillation seems energetically wasteful and unlikely to occur *in vivo*. Concern for the apparent energetic wastefulness of such oscillation may somewhat diminished by comparing the value of k_{cat} for robust ATPases (i.e. 100-1000 minute^{-1}; Graves Woodward et al., 1996, J. Biol. Chem. 271:13629-13635; Jiang et al., 1997, J. Biol. Chem. 272:7626-7632; Wong et al., 1996, Biochemistry 35:5726-5734)) to the value of k_{cat} for the hMSH2/hMSH6 complex in the presence of the G/T-mismatched DNA substrate (i.e. 26 minute^{-1}). Clearly, the hMSH2/hMSH6 complex would hydrolyze less ATP than robust ATPases, under the conditions described herein.

There are at least two other explanations of the physiological relevance of the enhanced ability of ATP to displace ADP bound to the complex. First, although the displacement rate is clearly faster in the presence of the G/T-mismatched DNA substrate than it is in the absence of DNA, the rate is still slow relative to the rate of mismatch repair events. Thus, the complex is able to complete few, if any, cycles of association with and dissociation from mismatched DNA and, as a result, hydrolyzes few, if any, ATP molecules. Alternately, the ADP-bound form of the complex when bound to mismatched DNA may be stabilized by other proteins that provide the ultimate trigger for displacement of complex-bound ADP by ATP during the course of a mismatch repair event. Regardless of the mechanism involved, it is clear from the observations presented in this Example that binding of the hMSH2/hMSH6 complex to the G/T-mismatched DNA substrate elicited a change in the protein such that, upon binding, the complex became competent to exchange ADP for ATP. In the absence of DNA, the complex could not exchange ADP for ATP.

Generality of MutS function

The studies described in this Example, which involved the human mismatch binding reaction catalyzed by the hMSH2/hMSH6 protein complex, are consistent with genetic studies performed in both bacteria and yeast. In those studies,

mutation of the adenine nucleotide binding and hydrolysis domain(s) resulted in a dominant mutator phenotype (Haber et al., 1991, EMBO. J. 10:2707-2715; Wu et al., 1994, J. Bacteriol 176:5393-5400; Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447). Those studies, combined with the studies described in this Example, indicate that there may be two opposing functional alterations of MutS homologs that can cause such a dominant mutator phenotype. First, alteration of the ability of the homolog to bind and/or exchange ADP for ATP can cause a dominant mutator phenotype. Second, alteration of the ability of the homolog to hydrolyze ATP can similarly cause such a phenotype. Inability of the homolog to bind to ADP or to exchange ADP for ATP would result in a permanently mismatched DNA-bound form of the MutS homolog. This form of the homolog would exclude the repair machinery from the mismatch site. Inability of the MutS homolog to hydrolyze ATP would result in a form of the homolog that would be unable to bind to mismatched DNA and which, therefore, would be unable to recruit the cellular mismatch repair proteins and factors to the site of the mismatch. Each these conditions would cause an increased mutation rate in the organism containing the homolog, as a consequence of the organism's depressed ability to repair mismatched DNA (Wu et al., 1994, J. Bacteriol 176:5393-5400).

Preliminary studies performed using the methods described herein and using purified *Escherichia coli* MutS protein suggest that *E. coli* MutS also functions as a molecular switch, albeit with a more stringent requirement for mismatch-induced nucleotide exchange. Therefore, the properties of the MutS homologs hMSH2 and hMSH6, as described herein appear to be properties of all MutS homologs, including, but not limited to, *E. coli* MutS, the human homologs hMSH2, hMSH3, and hMSH6.

Similarity of the hMSH2/hMSH6 complex to G-protein switches

The hMSH2/hMSH6 molecular switch is, in some respects, similar to G-protein switches which have been described (Bokoch et al., 1993, FASEB J. 7:750-759). G-proteins are known to trigger translocation events associated with protein synthesis (Laalami et al., 1996, Biochimie 78:577-589; Parmeggiani et al., 1981, Mol. Cell Biochem. 35:129-158), cascade events associated with cell signaling (Mederna et

al., 1993, Crit. Rev. Oncol. 4:615-661; Wiesmuller et al., 1994, Cell Signal 6:247-267) and physiological responses to ligand-binding by membrane receptors (Spiegel, 1987, Mol. Cell Endocrinol. 49:1-16). Many G-proteins are associated with regulators that stimulate both the GTPase activity of the G-protein (Tocque et al., 1997, Cell Signal 9:153-158) and the exchange of G-protein-bound GDP for GTP (Dohlman et al., 1997, J. Biol. Chem. 2 72:3871-3874; Quilliam et al., 1995, Bioessays 17:395-404). In fact, the Ras G-protein was determined to be unable to catalyze GTP hydrolysis because it is unable to exchange GDP for GTP. The discovery of a GTPase activating protein (GAP) that stimulated GTP γ-phosphate hydrolysis, and a guanine nucleotide exchange factor (GNEF) that stimulated the exchange of GDP for GTP, provided a model for regulation of the Ras G-protein switch (Tocque et al., 1997, Cell Signal 9:153-158; Dohlman et al., 1997, J. Biol. Chem. 2 72:3871-3874).

It has therefore been discovered that protein regulation of the excision-resynthesis processes associated with mismatch repair occurs by stimulation of the ATPase activity of the hMSH2/hMSH6 complex or of the ability of the complex to exchange ADP for ATP. The latter stimulation can occur either by stabilizing the ADP-bound form of the complex or by stimulating exchange of ADP for ATP to effect release of the complex from mismatched DNA. The human MutL homologs, hMLH1 performs these regulatory functions.

Example 2. A Mouse Construct Nullizygous for both the *msh2* and *p53* Genes and Methods of Making and Use Thereof

Transgenic mice which are nullizygous for both the *Msh2* gene and the *p53* gene have been made, and are referred to herein as *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. Other transgenic animals which are nullizygous for both the *Msh2* gene and the *p53* gene, and which particularly include mammals, especially including rodents such as mice and rats, may be made using methods analogous to those described herein and are useful in the screening methods described herein.

The development of female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos is phenotypically arrested at approximately the 9.5 day stage, and apoptosis is induced

shortly thereafter in the cells of these embryos. Male *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos are viable, but succumb to tumors significantly earlier than either *Msh2*$^{-/-}$ or *p53*$^{-/-}$ littermates (i.e. nullizygous *Msh2* mice or nullizygous *p53* mice, respectively). Furthermore, the frequency of microsatellite instability (MSI) in tumor tissue obtained from *Msh2*$^{-/-}$*p53*$^{-/-}$ mice is not significantly different than the frequency in tumor tissue obtained from *Msh2*$^{-/-}$ mice. Synergism in tumorigenesis and independent segregation of the MSI phenotype suggest that *Msh2* and *p53* are not genetically epistatic.

Msh2$^{-/-}$*p53*$^{-/-}$ mice are useful as models of disease or disorder states which cannot be identified in mice nullizygous for only one of the *Msh2* gene or the *p53* gene. Furthermore, *Msh2*$^{-/-}$*p53*$^{-/-}$ mice are useful for identifying compositions which affect the onset or progression of such a disease or disorder state. Thus, a *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse is particularly useful as a model system for studying multistep tumorigenesis.

As used herein, the term "nullizygous" refers to an animal which possesses a pair of null mutant alleles at a given genetic locus. Hence, a nullizygous *Xxx* mouse (wherein *Xxx* is any gene normally present in a mouse) does not possess a functional *Xxx* gene, whereas a wild-type mouse may possess one or two functional copies of the *Xxx* gene. To illustrate the notation used herein, the term "nullizygous *Xxx* mouse" is synonymous with the term "*Xxx*$^{-/-}$ mouse." Similarly, a "heterozygous *Xxx* mouse" has one functional *Xxx* allele and one non-functional *Xxx* allele, and is synonymous with the term "*Xxx*$^{+/-}$ mouse." A "wild type mouse" has at least one copy, and possibly two copies, of a functional *Xxx* allele, and is synonymous with the term "*Xxx*$^{+/\pm}$ mouse." A "homologous wild type mouse' has two copies of a functional *Xxx* allele, and is synonymous with the term "*Xxx*$^{+/+}$ mouse."

The materials and methods used in the experiments presented in this Example are now described.

Generation of *Msh2*$^{-/-}$*p53*$^{-/-}$ Mice

Methods for making heterozygous and nullizygous *Msh2* mice and heterozygous and nullizygous *p53* mice have been described (de Wind et al., 1995, Cell

82:321-330; Reitmair et al., 1995, Nat. Genet. 11:64-70; Donehower et al., 1992, Nature 356:215-221; Jacks et al., 1994, Curr. Biol. 4:1-7; Purdie et al., 1994, Oncogene 9:603-609).

Mice heterozygous for the *Msh2* gene (i.e. $Msh2^{+/-}p53^{+/+}$ mice) on a mixed C57BL/6J and 129/Ola background and mice heterozygous for the *p53* gene (i.e. $Msh2^{+/+}p53^{+/-}$ mice) on a mixed C57BL/6J and 129/Sv were mated to produce F1 progeny heterozygous for both genes (i.e. $Msh2^{+/-}p53^{+/-}$ mice). Heterozygous sibling F1 progeny were intercrossed to produce progeny nullizygous for both *Msh2* and *p53* (i.e. $Msh2^{-/-}p53^{-/-}$ mice). Mice were genotyped using *Msh2*- and *p53*- specific PCR-based assays, using methods well known in the art.

Isolation of Genomic DNA

Mouse genomic DNA was extracted from ear-notched tissue of mice and from amniotic tissue of mouse embryos at 9.5, 11.5, or 13.5 days of development, using a QIAamp Tissue Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

PCR-based Genotyping of Mice

A three-primer assay specific for *Msh2* was carried out as described (Reitmair et al., 1995, Nat. Genet. 11:64-70). A four-primer assay specific for *p53* was carried out using 50 ng of template DNA in a 50 µl reaction mixture containing 1 unit of *Taq* polymerase (Fisher Scientific, Malvern, PA) and 100 mM each of the following primers, each of which is identified with a five digit number and the sequence of each of which is listed:

10681 (5'-GTGTTTCATTAGTTCCCCACCTTGAC-3');

10480 (5'-ATGGGAGGCTGCCAGTCCTAACCC-3');

10588 (5'-GTGGGAGGGACAAAAGTTCGAGGCC-3'); and

10930 (5'-TTTACGGAGCCCTGGCGCTCGATGT-3').

The amplification reaction involved 35 cycles of amplification (94°C, 15 seconds; 56°C, 30 seconds; 72°C, 1 minute) using a Perkin-Elmer GeneAmp 9600 thermal cycler. The wild-type primers, *10681* and *10480*, amplified a product of about 320 bp

length, and the targeted allele (i.e. *p53*$^-$) primers, *10588* and *10930*, amplified a product of about 150 bp length.

The gender of embryos was determined using primers specific for the Y-chromosome gene as described (Sah et al., 1995, Nat. Genet. 10:175-180). The presence of the X-chromosome was confirmed separately in all cases using the following two X-chromosome specific primers to amplify the locus *DXMIT6*:

5'-ACCATTCAAATTGGCAAGG-3'; and

5'-GTGGCTCGAGTTGTTTGCAG-3'.

PCR cycling conditions were as described above for *p53* genotyping, except that the annealing temperature was 53°C, rather than 56°C. The X-chromosome specific primers amplified a product of about 210 bp in length. All PCR amplification products were resolved by electrophoresis on a 2% (w/v) agarose gel alongside a 100 bp polynucleotide ladder and were visualized by ethidium bromide staining.

Timed Pregnancies

Male and female mice that were *Msh2*$^{+/-}$*p53*$^{+/-}$, *Msh2*$^{+/-}$*p53*$^{-/-}$, or *Msh2*$^{-/-}$*p53*$^{+/-}$ were mated and each of the females was examined daily for the presence of a vaginal plug (an indicator of pregnancy which appears at about day 0.5 of embryo development). Pregnant females were sacrificed at 13.5 days, at 11.5 days, or at 9.5 days gestation. Embryos were dissected out from the pregnant females into Hank's Balanced Salt Solution (Gibco BRL, Grand Island, NY) under a dissecting microscope, fixed in 4% buffered formalin, and documented by photomicrography. Amnion was retrieved from each embryo, DNA extracted therefrom, and the sex and genotype of each embryo determined by PCR.

Histology

Tissue specimens were fixed in 10% (v/v) or 4% (v/v) buffered formalin and embedded in paraffin. Histological analysis was carried out on 3 micrometer-thick sections stained with hematoxylin and eosin (H&E).

TUNEL Assay

Paraffin-embedded tissue sections were dewaxed and rehydrated through a graded alcohol series, using methods well known in the art. Apoptotic cells and appropriate positive and negative control samples were analyzed using the *In Situ* Cell Detection Kit, AP with NBT/BCIP, manufactured by Boehringer Mannheim (Indianapolis, IN), according to the manufacturer's instructions. TUNEL-stained tissue sections were analyzed both by fluorescence and light microscopy.

Kaplan-Meier Survival

Kaplan-Meier survival probability was calculated for mice that were found dead or were sacrificed when found to be moribund. The age of the mice was calculated in days. Because no mice died in the control group, confidence limits could not be determined.

Microsatellite Instability in Lymphoid Tumors

Paired ear-notch (i.e. normal) and lymphoid tumor tissues were analyzed for microsatellite instability at five chromosomal loci: *D17Mit123*, *D10Mit2*, *D6Mit59*, *D4Mit27*, and *D3Mit203*. Microsatellite primer sequence pairs appropriate for amplification of these loci were obtained from the World Wide Web site of the Whitehead Institute for Genome Research (http://www.genome.wi.mit.edu), and were chosen to amplify fragments containing at least twenty dinucleotide repeat sequences. PCR amplifications were carried out in a total reaction volume of 25 µl, using 50 ng of DNA as template, 100 mM of each primer pair and 1 unit of *Taq* polymerase (Fisher). The amplification reaction involved 35 cycles of amplification (94°C, 15 seconds; 56°C, 30 seconds; 72°C, 1 minute). Amplified products were resolved by electrophoresis on a 6.7% (w/v) denaturing polyacrylamide gel and were visualized by silver nitrate staining of the gel.

The results of the studies described herein are now described.

Twenty-one $Msh2^{-/-}p53^{-/-}$ mice were generated from $Msh2^{+/-}p53^{+/-}$, $Msh2^{-/-}p53^{+/-}$, or $Msh2^{+/-}p53^{-/-}$ parents. When the gender of each of the twenty-one $Msh2^{-/-}p53^{-/-}$ mice was examined, all were determined to be male $Msh2^{-/-}p53^{-/-}$ mice. The absence of female $Msh2^{-/-}p53^{-/-}$ offspring is highly significant ($p < 0.001$) and is

unlikely to reflect the intrinsic bias for males observed in the colony corresponding to the mice, wherein the normal male:female ratio is 181:138.

The fertility of male *Msh2*$^{-/-}$*p53*$^{-/-}$ mice could not be determined, because they succumbed to tumors before they successfully mated. However, pathological examination of the testes of the male *Msh2*$^{-/-}$*p53*$^{-/-}$ mice did not reveal gross abnormalities upon autopsy, and histology revealed mature spermatogenesis in all twenty-one of the male *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. Taken together, these results suggest that *Msh2*$^{-/-}$*p53*$^{-/-}$ male mice are not likely to be sterile.

No gross morphological abnormalities were observed in *Msh2*$^{-/-}$ animals either *in utero* or post-natally (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nat. Genet. 11:64-70). In addition, the number of male and female *Msh2*$^{-/-}$ mice in the studies described herein was in accordance with the expected 1:1 ratio, which suggests that male and female nullizygous *Msh2* mice are equally viable. However, a decrease in the number of live born nullizygous *p53* mice from the expected Mendelian ratio was observed, which is qualitatively similar to previous reports, although our limited numbers did not indicate a sex bias (Sah et al., 1995, Nat. Genet. 10:175-180; Nicols et al., 1995, Nat. Genet. 10:181-187).

No female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice were found at weaning and none of thirteen one-day-old pups which were found dead in the litters of mating pairs were *Msh2*$^{-/-}$*p53*$^{-/-}$. Thus, all female embryos nullizygous for both *Msh2* and *p53* died *in utero*. To determine the point in embryonic development at which these embryos died, numerous timed pregnancies were established. Because *Msh2*$^{-/-}$*p53*$^{-/-}$ males were not available and *Msh2*$^{-/-}$*p53*$^{-/-}$ females were not viable, pairs of mice, each of which mice was a *Msh2*$^{+/-}$*p53*$^{+/-}$, *Msh2*$^{+/-}$*p53*$^{-/-}$, or *Msh2*$^{-/-}$*p53*$^{+/-}$ mouse, were mated to produce *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos. Pregnant females were sacrificed at 9.5, 11.5, and 13.5 days of gestation, the embryos were pathologically assessed for developmental defects and their genotype and gender were determined by PCR. The results of these analyses are presented in Table 1. A total of twenty-one embryos and six resorption sites were recovered from three females at day 13.5 of gestation. Of the twenty-one 13.5 day embryos, two male

Msh2$^{-/-}$*p53*$^{-/-}$ embryos and no female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos were recovered, although a total of five *Msh2*$^{-/-}$*p53*$^{-/-}$embryos were statistically expected. Two 13.5 day embryos (one male *Msh2*$^{+/-}$*p53*$^{-/-}$; one female *Msh2*$^{-/-}$*p53*$^{+/-}$) displayed exencephaly, while all other 13.5 day embryos appeared normal (Sah et al., 1995, Nat. Genet. 10:175-180).

Table 1. Sex and Morphological Phenotype of Timed Post-Implantation Embryos

Days Development	Resorption Sites	# of Embryos	Embryos Typed	Female *Msh2*$^{-/-}$*p53*$^{-/-}$		Male *Msh2*$^{-/-}$*p53*$^{-/-}$	
				Nor	Abnr	Nor	Abnr
e9.5	3	30	28	3	1	2	1
e11.5	11	21	17	0	4	2	0
e13.5	6	21	21	0	0	2	0
*28	-	*96	*96	*0	*0	*21	*0

Embryos that arrested in development, that were in resorption, or that displayed gross abnormalities were classified as abnormal (Abnr), while those embryos which were not arrested in development, were not in resorption, and did not display gross abnormalities were classified as normal (Nor). Thirteen newborn pups that were found dead, none of which were *Msh2*$^{-/-}$*p53*$^{-/-}$, are not represented in this Table.

*Refers to live-born animals at twenty-eight days following birth.

Twenty-one embryos and eleven resorption sites were recovered from three pregnant females at day 11.5 of gestation. Of these, complete PCR typing results were determined for seventeen embryos and one resorption site. Five embryos were determined to be *Msh2*$^{-/-}$*p53*$^{-/-}$, although eight *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos were statistically expected. Two of the five embryos were males that appeared morphologically normal (one is depicted in Fig. 1, Panel A), and three of the five embryos were females, all three of which had undergone developmental arrest, and all three of which are depicted in Fig. 1, Panels B, C, and D. The three female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos appeared opaque and somites were not visible. Based on the gross morphology of the three female *Msh2*$^{-/-}$

p53$^{-/-}$ embryos, it was calculated that they died at 9.5 days of development. The tissue from the resorption site was typed as female *Msh2*$^{-/-}$*p53*$^{-/-}$.

Thirty embryos and three resorption sites were recovered from pregnant females at day 9.5 of gestation. Twenty-eight embryos and one resorption site were successfully typed. Two embryos and a resorption site were found to be male *Msh2*$^{-/-}$*p53*$^{-/-}$, and four embryos were typed as female *Msh2*$^{-/-}$*p53*$^{-/-}$. Six *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos were statistically expected. Neither of the male *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos exhibited any gross morphological abnormality. It is likely that the male *Msh2*$^{-/-}$*p53*$^{-/-}$ resorption site represents a spontaneous abortion event. In one of the four female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos, the anterior neural tube was not closed and the heart was not seen to beat, which should occur around day 9 of development. These observations are consistent with a developmental delay that could result from late fertilization or implantation or alternatively, from a developmental abnormality that is apparent at day 9.5.

Paraffin embedded tissue sections from wildtype and *Msh2*$^{-/-}$ *p53*$^{-/-}$ female embryos, as depicted in Fig. 2, from *Msh2*$^{-/-}$ embryos, and from *p53*$^{-/-}$ embryos were examined at day 11.5 and at day 13.5. While the wildtype, *Msh2*$^{-/-}$, and *p53*$^{-/-}$ embryos had clearly distinguished developmental features at day 11.5, the arrested *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryos contained noncohesive cells without preservation of embryonal tissue structures. In addition, H&E stained *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryonic tissue sections appeared to contain an large number of "blebbed" structures typical of apoptotic cells. Furthermore, loss of nuclear hematoxylin stain typical for necrosis was not observed in H&E stained *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryonic tissue sections (Fig. 2, Panel B).

TUNEL staining was performed on the paraffin embedded tissue sections (Fig. 2, Panels C-F). Although wildtype (Fig. 2, Panels C and E), *Msh2*$^{-/-}$, and *p53*$^{-/-}$ embryos displayed circumscribed foci of apoptotic cells characteristic of normal embryonal development, *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryos displayed global catastrophic apoptosis (Fig. 2, Panels D and F). Furthermore,

fluorescence TUNEL staining of *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryos revealed a speckled intracellular patterning characteristic of fragmented chromatin (Fig. 2, Panel F). It was estimated that between about 60% and about 90% of cells in *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryos were undergoing visible apoptosis, as assessed by H&E and TUNEL staining.

Kaplan-Meier survival analysis was performed on a cohort of ninety-six mice, the data for which analysis are graphically depicted in Fig. 3. *Msh2*$^{-/-}$*p53*$^{-/-}$ mice began to die of generalized lymphomas at day 53 after birth and all twenty-one *Msh2*$^{-/-}$*p53*$^{-/-}$ mice were dead within four months of birth. In contrast, only 18% (eight of forty-four) of *Msh2*$^{-/-}$ littermates and 71% (five of seven) of *p53*$^{-/-}$ littermates were dead at the time the mice were analyzed. Thus, *Msh2*$^{-/-}$*p53*$^{-/-}$ mice had a significantly ($p<0.001$) reduced median survival time of 73 days compared with the median survival time of either *Msh2*$^{-/-}$ mice (i.e. 200 days) or *p53*$^{-/-}$ mice (i.e. 149 days). Furthermore, all twenty-four wild-type (i.e. *Msh2*$^{+/\pm}$*p53*$^{+/\pm}$) littermates were alive after approximately ten months. These results indicate that *Msh2* and *p53* null mutations cooperatively promote tumorigenesis. *p53* has also been shown to cooperate with a variety of other genes in mouse tumorigenesis models (Blyth et al., 1995, Oncogene 10:1717-1723; Williams et al., 1994, Cold Spring Harbor Symp. Quant. Biol. 59:449-457; Williams et al., 1994, Cell 79:329-339; Donehower et al., 1995, Genes Dev. 9:882-895; Nacht et al., 1996, Genes Dev. 10:2055-2066). However, as is apparent from Fig. 3, the effect on tumor-related death of having dual null mutations of *Msh2* and *p53* is greater than the sum of the effects of having a single null mutation in *Msh2* or *p53* alone. Thus, the *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse described herein has a phenotype which is significantly different from a mere combination of the phenotype of a *Msh2*$^{-/-}$ mouse and the phenotype of a *p53*$^{-/-}$ mouse.

Pathological examination of tumors showed that all twenty-one *Msh2*$^{-/-}$*p53*$^{-/-}$ mice developed highly aggressive generalized lymphomas

involving major organs. In addition, a pleomorphic sarcoma in the flank, a malignant fibrous histiocytoma of the neck, and a tubular adenoma of the small intestine were observed, while other epithelial neoplasms were not detected. The tumor spectrum of *Msh2*$^{-/-}$ and *p53*$^{-/-}$ mice appeared similar to previous observations (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nat. Genet. 11:64-70; Donehower et al., 1992, Nature 356:215-221; Jacks et al., 1994, Curr. Biol. 4:1-7; Purdie et al., 1994, Oncogene 9:603-609). The tumor spectrum of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice differs significantly from the tumor spectrum of either *Msh2*$^{-/-}$ or *p53*$^{-/-}$ mice. Thus, *Msh2*$^{-/-}$*p53*$^{-/-}$ mice have utility different from that of either *Msh2*$^{-/-}$ or *p53*$^{-/-}$ mice.

Normal and tumor tissues obtained from individual *Msh2*$^{-/-}$*p53*$^{-/-}$ mice were examined for microsatellite instability at five loci: *D17Mit123, D10Mit2, D6Mit59, D4Mit27*, and *D3Mit203* (Fig. 4). The results of these MSI studies are presented in Table 2. The frequency of MSI in tumor tissues obtained from *Msh2*$^{-/-}$ mice was not significantly different ($p>0.05$) the frequency of MSI in tumor tissues obtained from *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. Microsatellite instability was not observed in lymphomatous tumor tissue obtained from the seven *p53*$^{-/-}$ mice examined. The observation that *Msh2*$^{-/-}$*p53*$^{-/-}$ mice developed earlier onset of tumor-related disease, combined with the observed separate segregation of the MSI phenotype with the *Msh2* allele, suggests that *Msh2* and *p53* are not genetically epistatic.

Table 2. The Frequency of Microsatellite Instability in *p53*$^{-/-}$, *Msh2*$^{-/-}$, and *Msh2*$^{-/-}$*p53*$^{-/-}$ Mice.

Genotype Tumor / Normal Pairs	Tumors Examined (n)	MSI at ≥1 Locus	MSI at ≥2 Loci	MSI at ≥3 Loci
p53$^{-/-}$	7	0 (0%)	0 (0%)	0 (0%)
Msh2$^{-/-}$	8	6 (75%)	4 (50%)	3 (38%)
**Msh2*$^{-/-}$*p53*$^{-/-}$	21	17 (81%)	14 (67%)	12 (57%)

*Because female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice died during embryonic development, this refers to only male *Msh2*$^{-/-}$*p53*$^{-/-}$ mice.

It is remarkable that female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos underwent global developmental arrest and that widespread apoptosis of the cells of such embryos occurred around day 9.5 of development. That these embryos underwent implantation and gastrulation strongly suggests that they are capable of executing the earlier stages of embryogenesis. The arrested phenotype is reminiscent of that described for a small proportion of female *p53*$^{-/-}$ mice (Sah et al., 1995, Nat. Genet. 10:175-180). However, unlike *p53*$^{-/-}$ mice, no normal female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice or embryos were observed beyond 9.5 days of embryonic development. This observation supports the conclusion that the female embryonic lethality of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice is highly penetrant. In addition, none of the female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos displayed the exencephaly that characterized the *p53*$^{-/-}$ mice (Sah et al., 1995, Nat. Genet. 10:175-180). Furthermore, while there was no difference in apoptosis observed in developing *p53*$^{-/-}$ mouse embryos, global catastrophic apoptosis was clearly observed in all the *Msh2*$^{-/-}$*p53*$^{-/-}$ female mouse embryos examined. These results suggest that female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice succumb at an earlier stage and by an entirely different pathology than *p53*$^{-/-}$ mice.

Without being bound to any particular theory, the lethality observed in female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos is consistent with the following explanation. In the female embryonic lineage, dosage compensation

is achieved by random X chromosome inactivation around the time of gastrulation, at which time intense embryonic cellular proliferation and apoptosis promote embryonic differentiation (Lyon, 1961, Nature 190:372-373; Rastan, 1994, Curr. Opin. Genet. Dev. 4:292-297; Theiler, 1972, In: The House Mouse Development and Normal Stages from Fertilization to 4 Weeks of Age, Springer-Verlag, New York, p. 168). The global apoptotic effect need not occur coincidentally with X chromosome inactivation. The full effect of dysregulation may only become apparent after a number of cell divisions when the embryo undergoes a further burst of proliferation during embryonic 'turning' between 8 and 9.5 days.

It has been shown that the inactivated X chromosome replicates late in S phase (Taylor, 1960, J. Biophys. Biochem. Cytol. 7:455-464; Tagaki, 1974, Exp. Cell Res. 86:127-135). In addition, cells deficient in *p53* have been shown to be defective for damage-induced G_1/S checkpoint arrest, and cells that are deficient in MMR have been shown to be deficient for damage-induced G_2/M checkpoint arrest (Baker et al., 1990, Science 249:912-915; Diller et al., 1990, Mol. Cell. Biol. 10:5772-5781; Lin et al., 1992, Proc. Natl. Acad. Sci. USA 89:9210-9214; Hawn et al., 1995, Canc. Res. 55:3721-3725; Marra et al., 1996, Oncogene 13:2189-2196). Thus, female-specific *Msh2*$^{-/-}$*p53*$^{-/-}$ embryo lethality may result from dysregulation of damage-induced arrest checkpoint control, wherein such dysregulation is caused by a deficiency of both *p53* and *Msh2*, and whereby such dysregulation results in an inability of *Msh2*$^{-/-}$*p53*$^{-/-}$ cells to arrest cell division and repair damage introduced into the late replicating inactive X chromosome. Such damage could take the form of nonreplicated regions or chromosomal fragments that have resulted from inappropriate cell division prior to the completion of inactive X chromosome replication. Fragmented, reactivated, or otherwise altered inactive X chromosomes may then lead to global catastrophic cellular failure, developmental arrest, and apoptosis. Furthermore, the observation that the

highest levels of p53 mRNA are detected in wild-type embryos between 9 and 11 days of development suggests an important role for p53 protein within this time frame (Rogel et al., 1985, Mol. Cell. Biol. 5:2851-2855).

Screening Methods

A standard screening procedure is now described which is useful for determining the tumorigenetic potential of a compound. *Msh2*$^{-/-}$*p53*$^{-/-}$ mice are generated as described herein. While this procedures is described with respect to particular protocols and mice, it will be appreciated that the screening procedure described should not be construed to limit the invention in any way.

A predetermined amount of the compound is administered to the *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse by any practical means. The method of administration of the compound is not critical. By way of example, the compound may be administered orally, intraperitoneally, intravenously, topically, intramuscularly, or via a pulmonary route.

To reduce any potential for bias, the study is blinded. A first investigator treats all mice with compound(s) and identifiably marks or cages the transgenic mice, so that the nature of the treatments will not be known to a second investigator, who performs all tumor counts, weighing, and general observations.

Following administration of the compound, the *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse, each *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse is observed for about four months. Each mouse is examined approximately daily. Every week, each mouse is weighed, observed for any clinically-relevant symptoms, and the number and extent of tumors are assessed.

After observations are completed, the rate of tumor incidence and the tumor yield are determined for each group of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice to which the compound was applied. A higher or lower rate of tumor incidence or a higher or lower tumor yield for a group of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice to which the

compound was applied, compared with the levels of tumor incidence and tumor yield for a group of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice to which the compound was not applied, is an indication that the compound affects tumorigenesis.

Preferably, groups of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice are used, with each mouse in a group being treated identically. Also preferred are studies in which one of at least three different dose levels of the compound are applied to the mice in each of at least three corresponding groups of transgenic mice. It is preferred, where possible, to demonstrate a statistically significant difference ($P < 0.05$) between the average rate of tumor incidence or the average tumor yield for the first dose level and the average rate of tumor incidence or the average tumor yield for the third dose level.

Msh2$^{-/-}$*p53*$^{-/-}$ mice may also be used to identify the ability of a compound to affect apoptosis. This assay is performed identically to the tumorigenesis assay described herein, except that the compound is administered to *Msh2*$^{-/-}$*p53*$^{-/-}$ mice *in utero*, and the embryonic development of female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice is assessed, rather than tumor incidence or yield. A difference between embryonic development in female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos to which the compound was administered and embryonic development in female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos to which the compound was not administered is an indication that the compound affects apoptosis.

Msh2$^{-/-}$*p53*$^{-/-}$ mice may also be used to identify the ability of a compound to affect the aging process. This assay is performed identically to the apoptosis assay described herein, except that a difference between embryonic development in female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos to which the compound was administered and embryonic development in female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos to which the compound was not administered is an indication that the compound affects the aging process.

Methods of making a cell line from a cell of a nonhuman animal are well known in the art. A cell line which is nullizygous for both the *Msh2*

gene and the *p53* gene can be made by culturing a cell obtained from the nonhuman animal of the invention.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

What is claimed is:

1. A method of binding a MutS homolog to a mismatched region of a duplex DNA molecule, the method comprising contacting said homolog with said duplex DNA molecule in the presence of a solution comprising an ADP molecule, whereby said homolog binds to said mismatched region.

2. The method of claim 1, wherein said homolog is selected from the group consisting of the *E. coli* MutS protein, an hMSH2 protein dimer, a complex comprising an hMSH2 protein molecule and either an hMSH3 protein molecule or an hMSH6 protein molecule, a yeast MSH2 protein dimer, a complex comprising a yeast MSH2 protein molecule and either a yeast MSH3 protein molecule or a yeast MSH6 protein molecule, a dimer of a rat homolog of hMSH2, a dimer of a *Xenopus* homolog of hMSH2, a dimer of a *Drosophila* homolog of hMSH2, a dimer of a murine homolog of hMSH2, and a complex comprising a murine homolog of hMSH2 and either a murine homolog of hMSH3 or a murine homolog of hMSH6.

3. The method of claim 2, wherein said homolog comprises a complex comprising an hMSH2 protein molecule and an hMSH6 protein molecule.

4. The method of claim 1, wherein said solution comprises at least about 20 micromolar ADP.

5. The method of claim 1, wherein said solution comprises less than about 5 micromolar ATP.

6. The method of claim 1, wherein said solution further comprises ATP and wherein the ratio of the concentration of ADP in said solution to the ratio of the concentration of ATP in said solution is greater than about two.

7. The method of claim 6, wherein said solution is substantially free of ATP.

8. The method of claim 1, wherein said duplex DNA molecule is suspended in said solution.

9. The method of claim 1, wherein said homolog is suspended in said solution.

10. The method of claim 9, wherein said duplex DNA molecule is suspended in said solution.

11. A method of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules, the method comprising

providing a MutS homolog and a solution comprising an ADP molecule and said population of DNA molecules, whereby said homolog binds to said mismatched region, and

segregating said homolog from said population, whereby said duplex DNA molecule is segregated from said population.

12. The method of claim 11, wherein said homolog is bound to a solid support and wherein segregating said homolog from said population comprises rinsing said solid support with a solution which does not comprise said population.

13. The method of claim 11, wherein said population comprises a plurality of cDNA molecules, wherein each of said cDNA molecules is made by reverse transcription of an RNA molecule obtained from an organism.

14. The method of claim 13, wherein said organism is a mammal.

15. The method of claim 14, wherein said mammal is a human.

16. The method of claim 11, further comprising contacting said homolog with a solution comprising an ATP molecule after segregating said

homolog from said population, whereby said mismatched region dissociates from said homolog.

17. A method of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence, the method comprising

annealing a first single-stranded DNA molecule and a second single-stranded DNA molecule to form a duplex DNA molecule, wherein said first molecule has said sample sequence and wherein said second molecule has a nucleotide sequence which is complementary to said reference sequence, whereby if there is a difference between said sample sequence and said reference sequence then said duplex DNA molecule has a mismatched region,

thereafter contacting said duplex DNA molecule and a MutS homolog in the presence of a solution comprising an ADP molecule, whereby said homolog binds specifically to said duplex DNA molecule if said duplex DNA molecule comprises said mismatched region, and

thereafter determining whether said homolog binds specifically to said duplex DNA molecule, whereby specific binding of said homolog to said duplex DNA molecule is an indication that there is a difference between said sample nucleotide sequence and said reference nucleotide sequence.

18. The method of claim 17, wherein said sample nucleotide sequence comprises the sequence of a region of a gene obtained from a first organism and wherein said reference nucleotide sequence comprises the sequence of said region obtained from a second organism.

19. The method of claim 18, wherein said first organism and said second organism are the same species.

20. The method of claim 19, wherein each of said first organism and said second organism is a human.

21. The method of claim 17, wherein said sample nucleotide sequence comprises the sequence of a region of a gene obtained from an

organism and wherein said reference nucleotide sequence comprises a consensus nucleotide sequence of said region.

22. The method of claim 21, wherein said gene is the human *msh2* gene.

23. A kit for separating a mismatched duplex DNA molecule from a population of molecules, the kit comprising

an MutS homolog;

a linker for binding said homolog to a solid support; and

an ADP molecule.

24. A method of detecting a predisposition of a mammal to carcinogenesis, the method comprising

annealing a first single-stranded DNA molecule and a second single-stranded DNA molecule to form a duplex DNA molecule, wherein said first molecule has the nucleotide sequence of a region of a mammalian gene associated with carcinogenesis and wherein said second molecule has a nucleotide sequence which is complementary to the consensus nucleotide sequence of said region, whereby if there is a sequence difference between said first molecule and said second molecule then said duplex DNA molecule has at least one mismatched region,

thereafter contacting said duplex DNA molecule and a MutS homolog in the presence of a solution comprising an ADP molecule, whereby said homolog binds to said duplex DNA molecule if said duplex DNA molecule comprises a mismatched region, and

thereafter determining whether said homolog binds to said duplex DNA molecule, whereby binding of said homolog to said duplex DNA molecule is an indication of said predisposition of said mammal to carcinogenesis.

25. The method of claim 24, wherein the mammalian gene associated with carcinogenesis is selected from the group consisting of the

msh2 gene, the *msh3* gene, the *msh6* gene, the *mlh1* gene, the *pms2* gene, the *brca1* gene, the *brca2* gene, the *pten* gene, and the *p53* gene.

26. The method of claim 24, wherein said mammalian gene is the human *msh2* gene and wherein said predisposition of a mammal for carcinogenesis is predisposition of a human for hereditary non-polyposis colorectal cancer.

27. A method of fractionating a population of duplex DNA molecules comprising

contacting said population with a MutS homolog in the presence of ADP, whereby said homolog is capable of binding to a duplex DNA molecule of said population if said duplex DNA molecule comprises a mismatched region,

segregating said homolog from said population, and

contacting said homolog with a dissociation solution comprising an ATP molecule, whereby said duplex DNA molecule dissociates from said homolog, whereby said population is fractionated.

28. A method of selectively amplifying at least one mismatched duplex DNA molecule of a population of duplex DNA molecules, the method comprising

contacting said population with a MutS homolog in the presence of a solution comprising an ADP molecule, whereby said homolog binds to said at least one mismatched duplex DNA molecule,

thereafter segregating said homolog from said population, whereby each said at least one mismatched duplex DNA molecule is segregated from said population, and

thereafter amplifying each said at least one mismatched duplex DNA molecule using a PCR technique, whereby each of said at least one mismatched duplex DNA molecule is selectively amplified.

29. A method of detecting the presence of a genetic polymorphism in the genome of an animal comprising

amplifying a region of each of two copies of a gene of said animal to yield an amplified first copy and an amplified second copy,

thereafter mixing and denaturing each of said first copy and said second copy to yield a first mixture of nucleic acids comprising a first sense copy, a first antisense copy, a second sense copy, and a second antisense copy,

thereafter annealing the nucleic acids in said first mixture to yield a second mixture of nucleic acids comprising said first copy, said second copy, a first sense-second antisense duplex DNA molecule, and a second sense-first antisense duplex DNA molecule, whereby if said first sense copy and said second antisense copy are not completely complementary then said first sense-second antisense duplex DNA molecule will have a first mismatched region, and whereby if said second sense copy and said first antisense copy are not completely complementary then said second sense-first antisense duplex DNA molecule will have a second mismatched region,

thereafter contacting said second mixture with a MutS homolog in the presence of a solution comprising an ADP molecule, whereby said homolog is capable of binding to either of said first mismatched region or said second mismatched region,

thereafter segregating said homolog from said second mixture, and

thereafter detecting the presence or absence of either of said first sense-second antisense duplex DNA molecule or said second sense-first antisense duplex DNA molecule, whereby the presence of either of said first sense-second antisense duplex DNA molecule or said second sense-first antisense duplex DNA molecule is an indication that said animal has a genetic polymorphism.

30. A composition for segregating a mismatched duplex DNA molecule from a population of molecules, said composition comprising a MutS

homolog bound to a solid support, wherein said support is in liquid contact with a solution comprising an ADP molecule.

31. A kit for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence, said kit comprising

a pair of primers adjacent said region for amplifying said region;

a duplex DNA molecule having said reference nucleotide sequence;

a solid support;

a MutS homolog;

a linker for binding said MutS homolog to said solid support; and

an ADP molecule.

32. A nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene.

33. The nonhuman mammal of claim 32, wherein said mammal is a rodent.

34. The nonhuman mammal of claim 33, wherein said nonhuman mammal is a mouse.

35. The nonhuman mammal of claim 32, wherein said mammal has an average survival time shorter than the average survival time of a nullizygous *Msh2* mammal of the same species.

36. The nonhuman mammal of claim 32, wherein said mammal has an average survival time shorter than the average survival time of a nullizygous *p53* mammal of the same species.

37. The nonhuman mammal of claim 32, wherein said mammal exhibits female-specific embryonic lethality.

38. A method of making a nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene, said method comprising mating a first mammal of a species, said first mammal comprising at least one null allele of the *Msh2* gene of said species with a second mammal of said

species, said second mammal comprising at least one null allele of the *p53* gene of said species.

39. A method of identifying a compound which affects tumorigenesis in a mammal comprising

administering said compound to a first nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene, and

comparing tumor incidence in said first transgenic mammal with tumor incidence in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered said compound, wherein a difference in tumor incidence in said first transgenic mammal compared with tumor incidence in said second transgenic mammal is an indication that said compound affects tumorigenesis in said mammal.

40. A method of identifying a compound which affects apoptosis in a mammal comprising

administering said compound *in utero* to a first nonhuman mammal embryo which is nullizygous for both the *Msh2* gene and the *p53* gene, and

comparing embryonic development in said first transgenic mammal embryo with embryonic development in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered said compound, wherein a difference in embryonic development of said first transgenic mammal compared with embryonic development of said second transgenic mammal is an indication that said compound affects apoptosis in said mammal.

41. A method of identifying a compound which affects the aging process in a mammal comprising

administering said compound *in utero* to a first nonhuman mammal embryo which is nullizygous for both the *Msh2* gene and the *p53* gene, and

comparing embryonic development in said first transgenic mammal embryo with embryonic development in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered said compound, wherein a difference in embryonic development of said first transgenic mammal compared with embryonic development of said second transgenic mammal is an indication that said compound affects the aging process in said mammal.

42. A cell line which is nullizygous for both the *Msh2* gene and the *p53* gene, wherein said cell line is made by culturing a cell obtained from the nonhuman mammal of claim 32.

ABSTRACT OF THE DISCLOSURE

Compositions, and products comprising a MutS homolog which binds to a mismatched region of a duplex DNA molecule in the presence of ADP are provided, as are methods of bindinging MutS homologs to mismatched DNA in the presence of ADP. Nonhuman mammals which are nullizygous for both the *Msh2* gene and the *p53* gene are also provided, as are methods of making and using the same.

Attorney's Docket No. 9855-6 (OTT-3026)

Applicant or Patentee: **Fishel *et al.***
Application or Patent No.: **Not Yet Assigned**
Filed or Issued: **Herewith**
For: **ADP-ACTIVATION OF DNA MISMATCH BINDING PROTEINS**

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

NAME OF ORGANIZATION: **Thomas Jefferson University**
ADDRESS OF ORGANIZATION: 1020 Walnut Street
Philadelphia, PA 10107

TYPE OF ORGANIZATION:

[X] University or other institution of higher education.
[ ] Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)).
[ ] Nonprofit scientific or educational under statute of state of the United States of America.
Name of State _____
Citation of Statute _____
[ ] Would qualify as tax exempt under Internal Revenue Code (26 USC 501(a) and 501(c)(3) if located in the United States of America.
[ ] Would qualify as nonprofit scientific or educational under statute of state of United States of America if located in the United States of America.
Name of State _____
Citation of Statute _____

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, with regard to the invention entitled **ADP-ACTIVATION OF DNA MISMATCH BINDING PROTEINS** by inventor **Fishel *et al.*** described in

[X] the specification filed herewith.
[ ] Application No. _____
Filed _____
[ ] Patent No. _____
Issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual concern or organization having rights to the invention is listed below* and no rights to the invention are held

by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27).

FULL NAME _____
ADDRESS _____

[ ]Individual [ ]Small Business Concern [ ]Nonprofit Organization

FULL NAME _____
ADDRESS _____

[ ]Individual [ ]Small Business Concern [ ]Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in the status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Abram M. Goldfinger, M.B.A.
TITLE IN ORGANIZATION Director of the Office of Technology Transfer
ADDRESS OF PERSON SIGNING 1020 Locust Street
Philadelphia, PA 19107-6799

SIGNATURE _____ DATE: 11/25/97

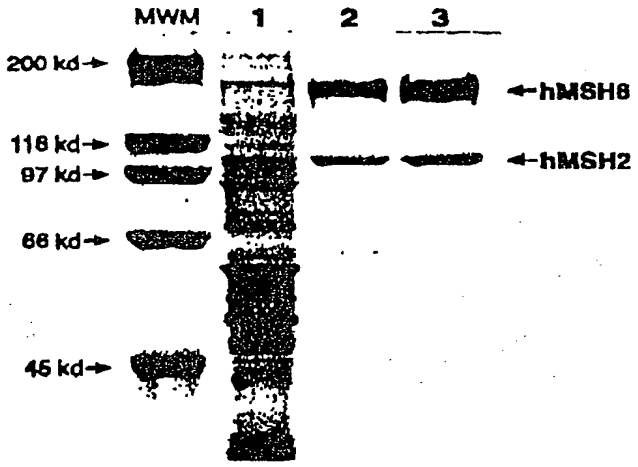
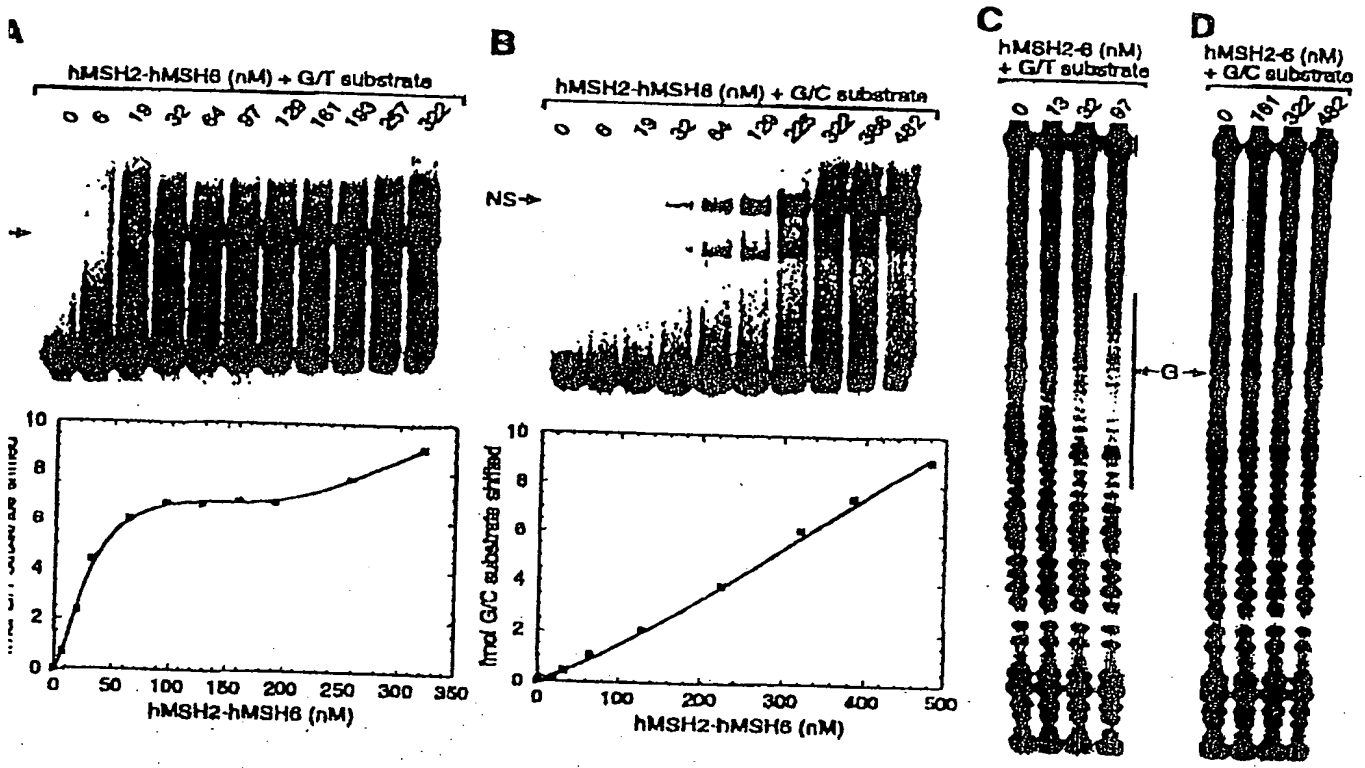


FIGURE 1


A
hMSH2-hMSH6 (nM) + G/T substrate
0 6 16 32 64 87 128 161 193 257 322
B
hMSH2-hMSH6 (nM) + G/C substrate
0 6 16 32 64 128 257 387 451
NS
fmol G/C substrate shifted
hMSH2-hMSH6 (nM)
C
hMSH2-6 (nM)
+ G/T substrate
0 13 32 67
G
D
hMSH2-6 (nM)
+ G/C substrate
0 161 322 482

E F

FIGURE 2

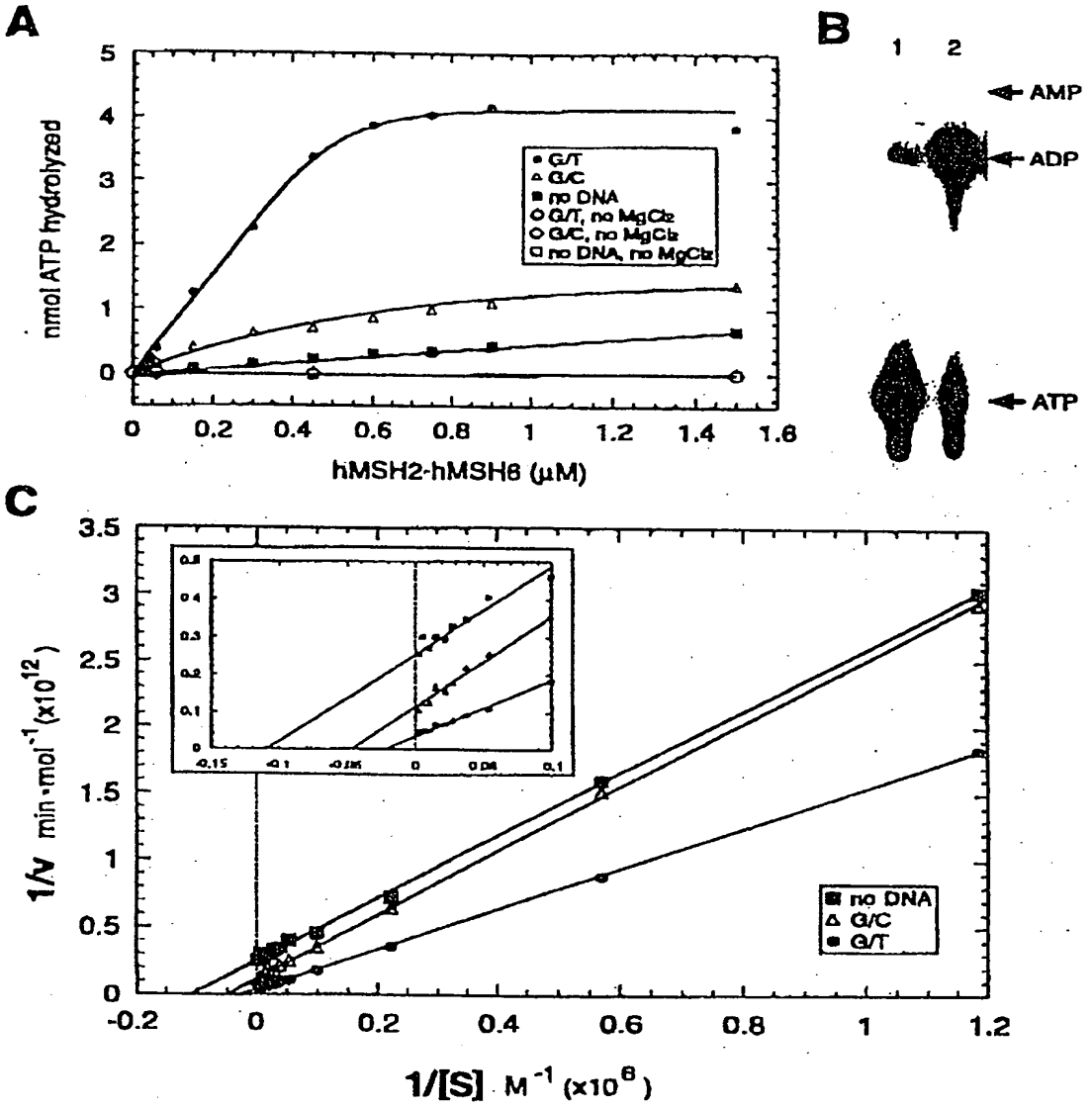

A
nmol ATP hydrolyzed
hMSH2-hMSH6 (μM)
G/T
G/C
no DNA
G/T, no MgCl2
G/C, no MgCl2
no DNA, no MgCl2
B
1
2
AMP
ADP
ATP
C
1/v min·mol-1 (x10^12)
1/[S] M-1 (x10^6)
no DNA
G/C
G/T

FIGURE 3

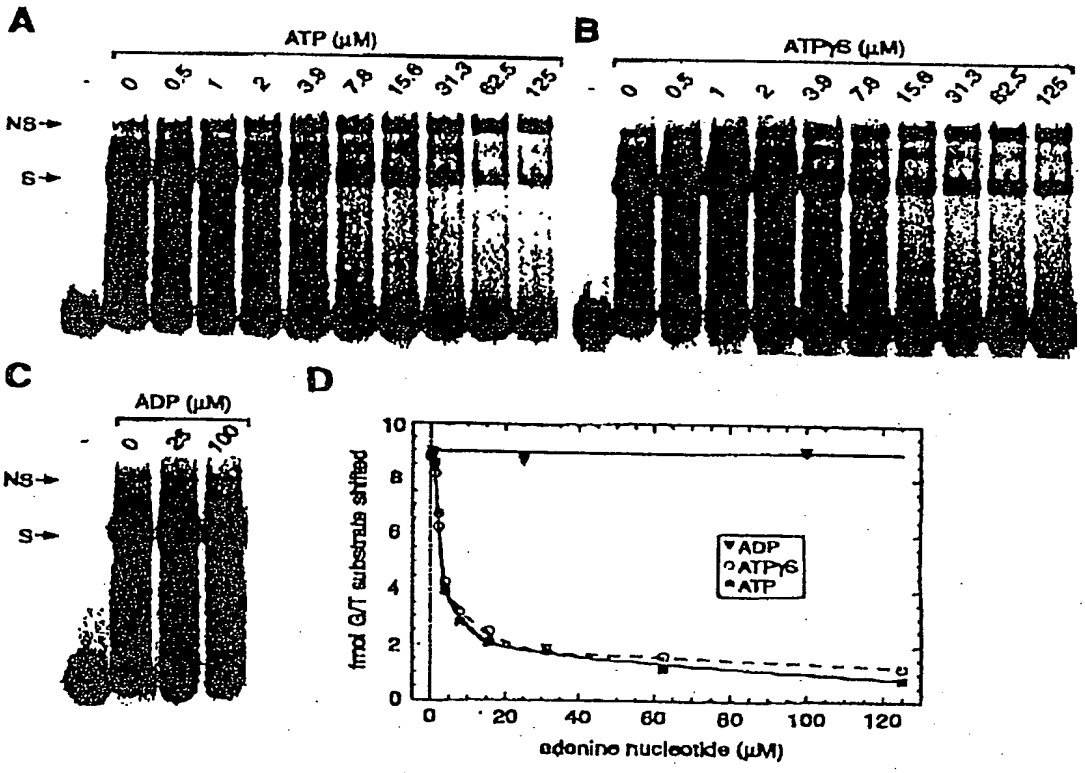

A
ATP (μM)
- 0 0.5 1 2 3.9 7.8 15.6 31.3 62.5 125
NS
S
B
ATPγS (μM)
- 0 0.5 1 2 3.9 7.8 15.6 31.3 62.5 125
C
ADP (μM)
- 0 25 100
NS
S
D
fmol G/T substrate shifted
10
8
6
4
2
0
0 20 40 60 80 100 120
adenine nucleotide (μM)
ADP
ATPγS
ATP

FIGURE 4

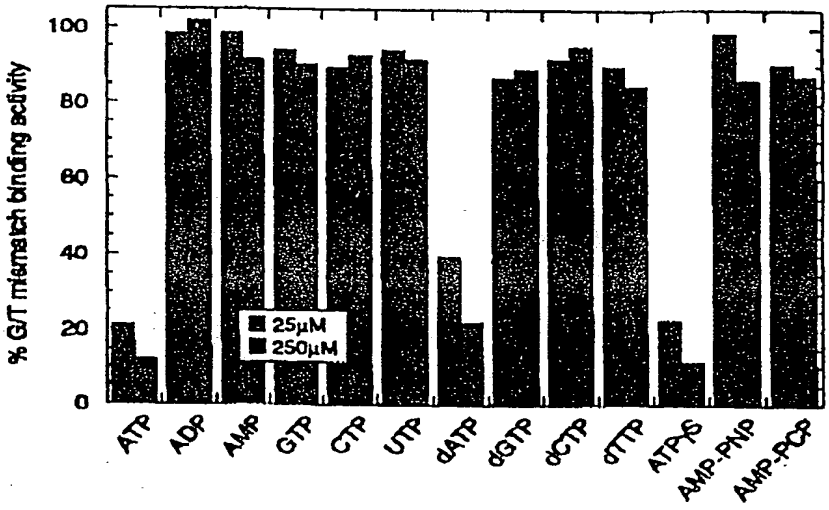

% G/T mismatch binding activity
100
80
60
40
20
0
25μM
250μM
ATP
ADP
AMP
GTP
CTP
UTP
dATP
dGTP
dCTP
dTTP
ATP-S
AMP-PNP
AMP-PCP

FIGURE 5

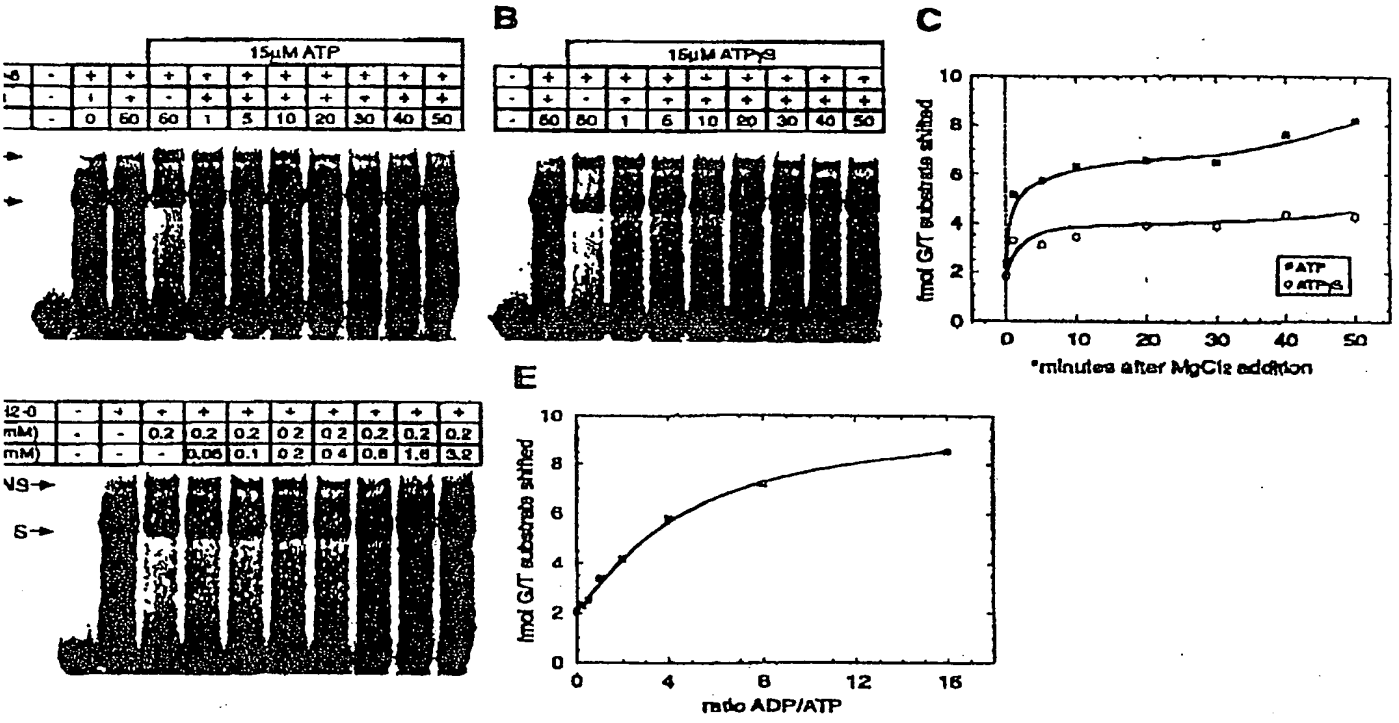

B
C
15µM ATP
15µM ATPγS
fmol G/T substrate shifted
*minutes after MgCl2 addition
*ATP
oATPγS
E
fmol G/T substrate shifted
ratio ADP/ATP

FIGURE 6

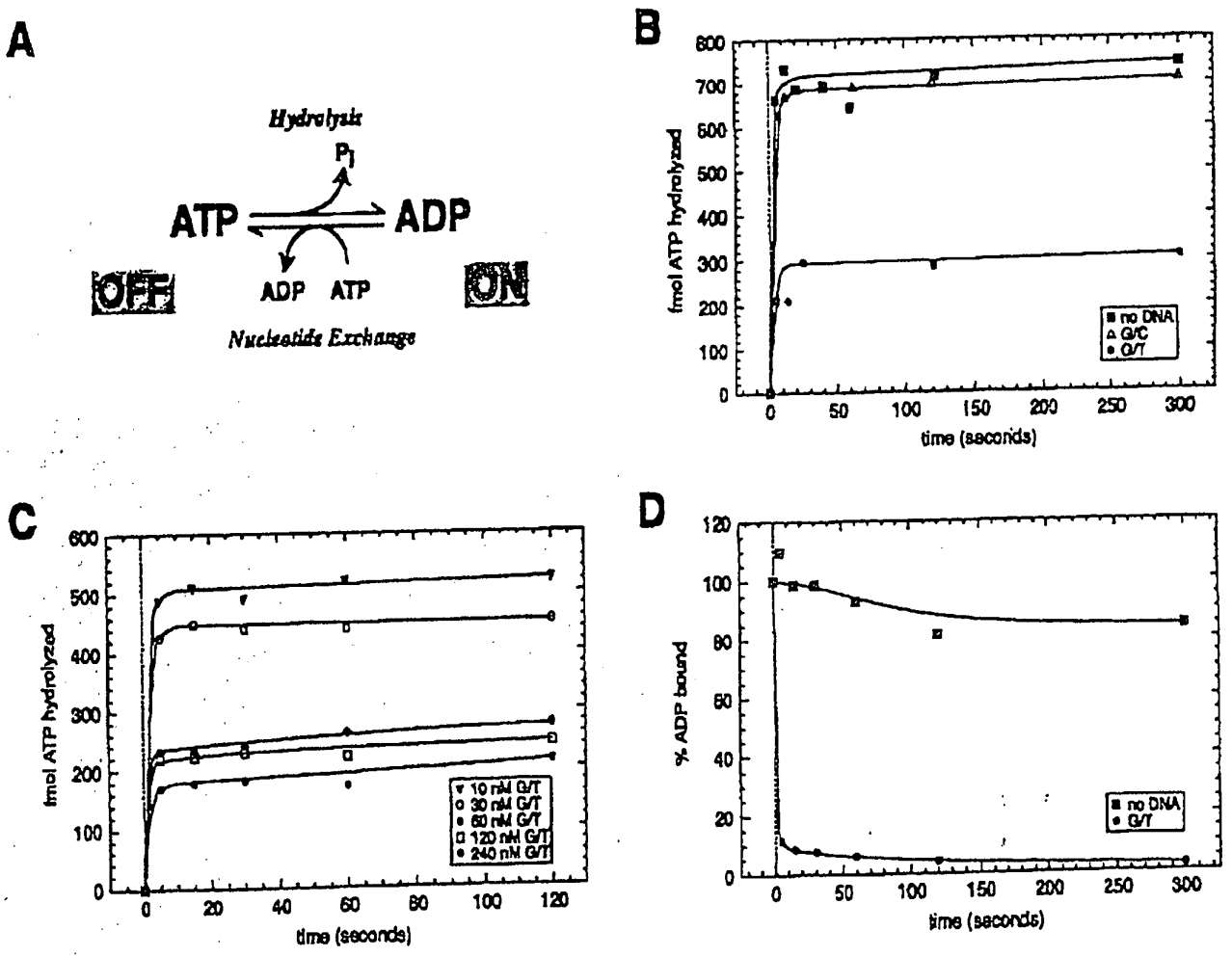

A
Hydrolysis
P_i
ATP
ADP
OFF
ADP ATP
ON
Nucleotide Exchange
B
fmol ATP hydrolyzed
time (seconds)
no DNA
G/C
G/T
C
fmol ATP hydrolyzed
time (seconds)
10 nM G/T
30 nM G/T
60 nM G/T
120 nM G/T
240 nM G/T
D
% ADP bound
time (seconds)
no DNA
G/T

FIGURE 7

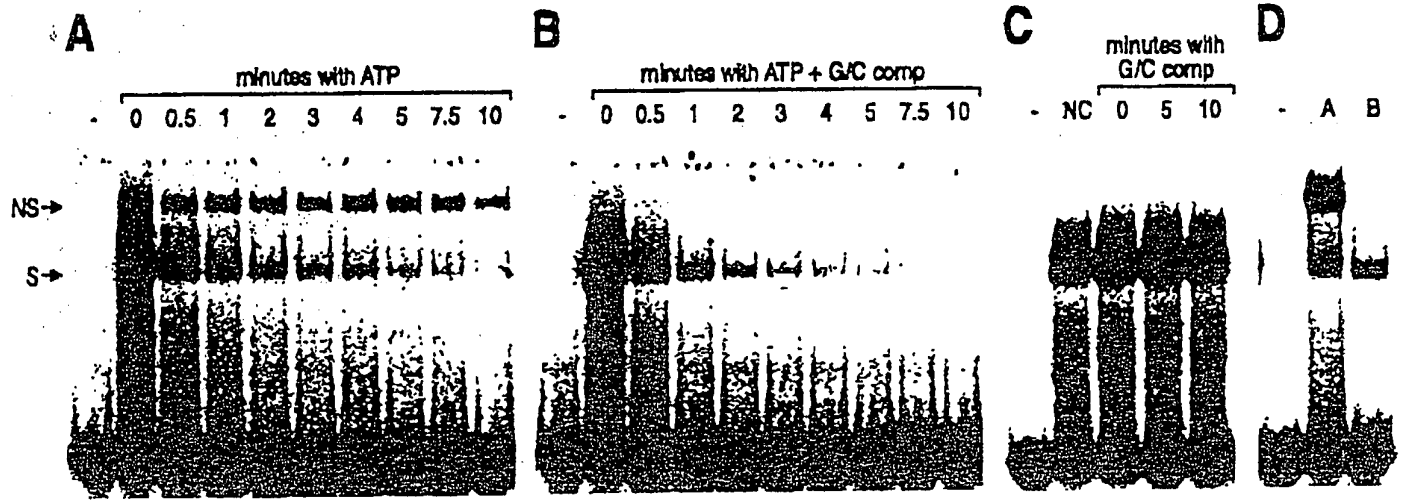

A
minutes with ATP
- 0 0.5 1 2 3 4 5 7.5 10
NS→
S→
B
minutes with ATP + G/C comp
- 0 0.5 1 2 3 4 5 7.5 10
C
minutes with G/C comp
- NC 0 5 10
D
- A B

FIGURE 8

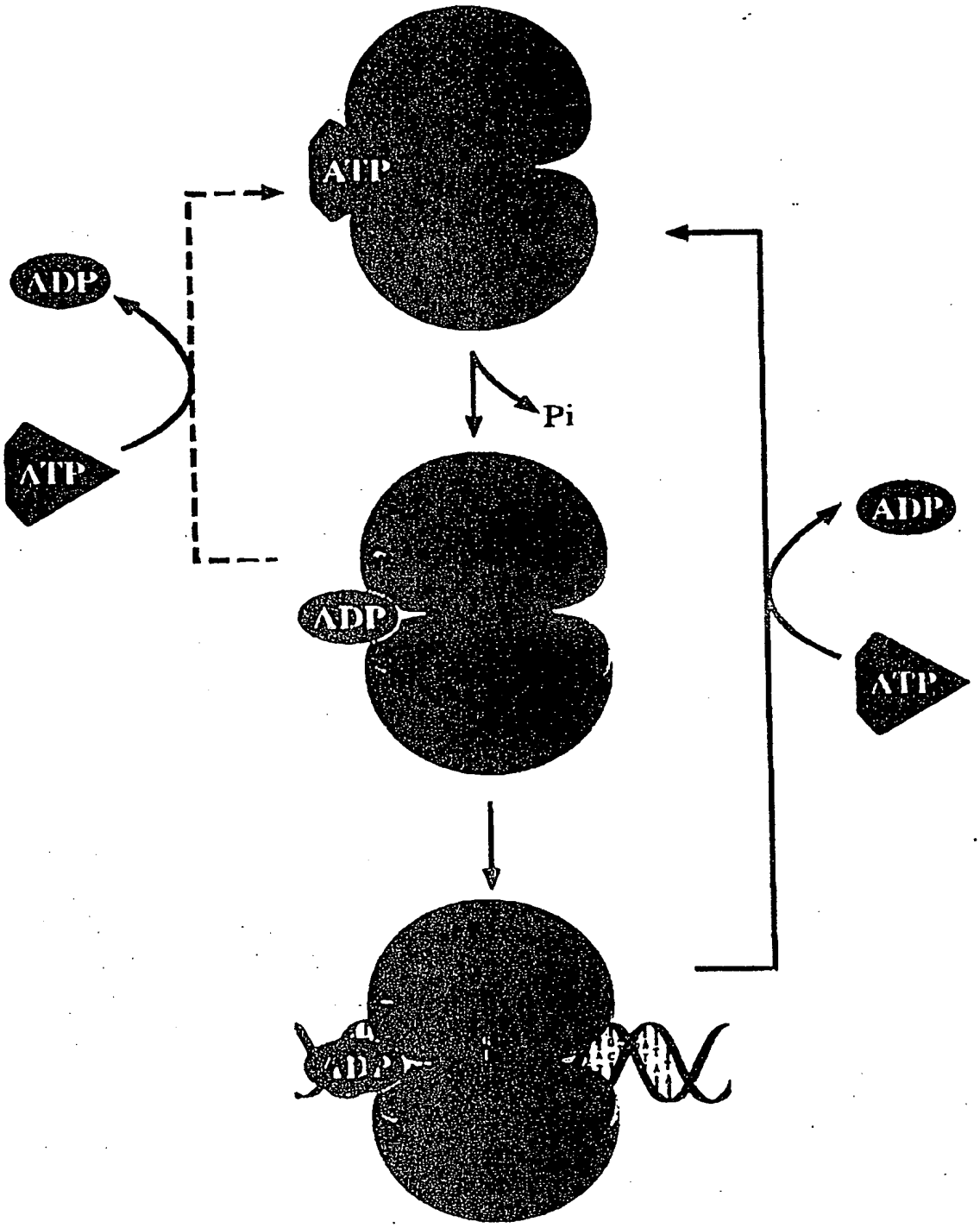


FIGURE 9

SEQ ID NO:1

5' CGGCGAATTC CACCAAGCTT GATCGCTCGA GGTACCAGG 3'

SEQ ID NO: 2

5' CCTGGTACCT CGAGCGATCA AGCTTGGTGG AATTCGCCG 3'

SEQ ID NO: 3

5' CCTGGTACCT CGAGCGATCG AGCTTGGTGG AATTCGCCG 3'

SEQ ID NO:4

5' AAAGCTGGAG CTGAAGCTTA GCTTAGGATC ATCGAGGATC
GAGCTCGGTG CAATTCAGCG GTACCCAATT CGCCCTATAG T 3'

SEQ ID NO: 5

5' ACTATAGGGC GAATTGGGTA CCGCTGAATT GCACCGAGCT
CGATCCTCGA TGATCCTAAG CTAAGCTTCA GCTCCAGCTT T 3'

SEQ ID NO: 6

5' ACTATAGGGC GAATTGGGTA CCGCTGAATT GCACCGAGCT
TGATCCTCGA TGATCCTAAG CTAAGCTTCA GCTCCAGCTT T 3'

FIGURE 10

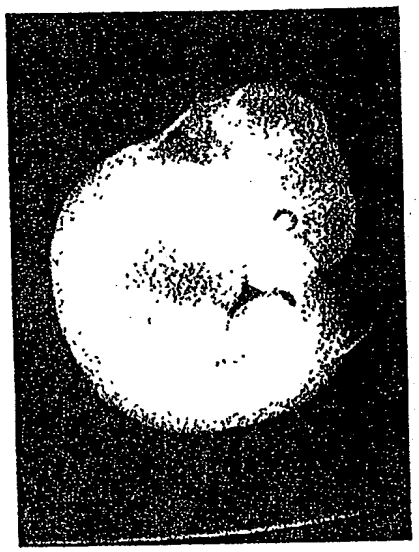


FIGURE 11A

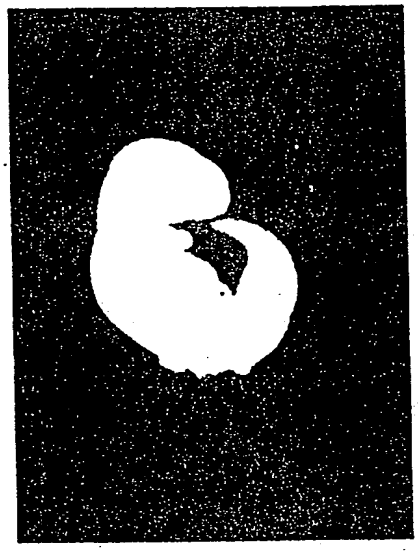


FIGURE 11B

FIGURE 11C

FIGURE 11D

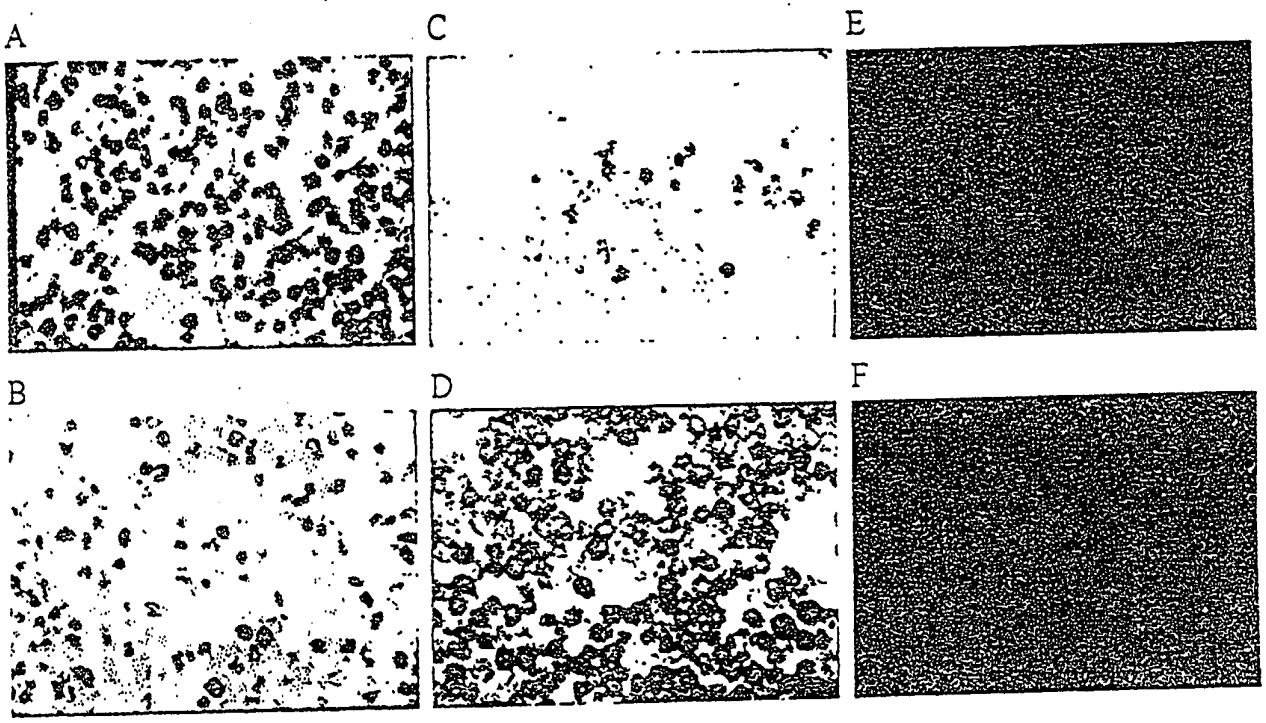


FIGURE 12

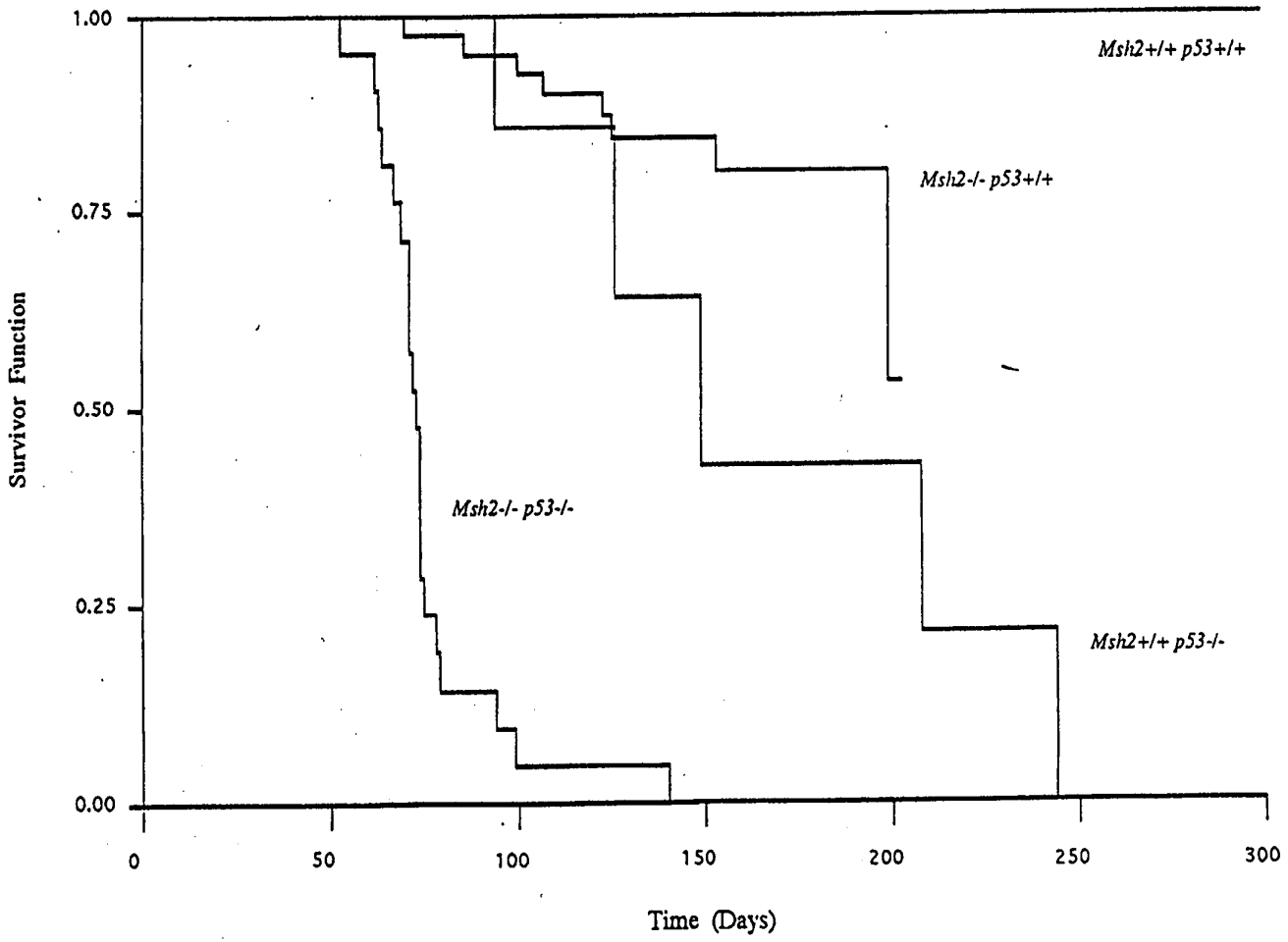

1.00
0.75
0.50
0.25
0.00
Survivor Function
0
50
100
150
200
250
300
Time (Days)
Msh2+/+ p53+/+
Msh2-/- p53+/+
Msh2-/- p53-/-
Msh2+/+ p53-/-

FIGURE 13

FIGURE 14

Appendix C A/PROV **PATENT**

"EXPRESS MAIL" MAILING LABEL NO.: EL004277770US

BOX PROVISIONAL APPLICATION

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (c).

Attorney Docket Number: 9855-7U1 (OTT-3026) Type a plus sign (+) inside this box: [+]

INVENTOR(s) APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Fishel	Robert		Penn Valley, PA
Gradia	Scott		Philadelphia, PA
Bocker	Tina		Philadelphia, PA
Acharya	Samir		Philadelphia, PA
Cranston	Aaron		Philadelphia, PA

TITLE OF INVENTION (280 characters maximum)

COMPOSITIONS AND METHODS FOR EFFECTING AND REGULATING ADP-ACTIVATION OF DNA MISMATCH BINDING PROTEINS

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS (*check all that apply*)

[X] Specification - *Number of Pages:* 94
[X] Drawing(s) - *Number of Sheets:* 17
[X] Small Entity Statement
[ ] Other (*specify*):

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (*check one*)

[X] The Commissioner is hereby authorized to charge filing fees or credit Deposit Account Number: 16-0235 (*Attach duplicate copy of this page if paying by deposit account*).

[X] PROVISIONAL FILING FEE AMOUNT: $75

The invention was made by an agency of the United States Government or under a contract with an agency of the U.S. Government.

[ ] No, or [X] Yes, the name of the U.S. Government agency and the Government contract number are: NIH Grant Nos. CA56542 and CA67007 and NRSA Grant No. CA73134

Respectfully submitted,

July 23, 1998
(Date)

By: [signature]
Gary D. Colby, Ph.D., J.D.
Registration No. 40,961

[ ] Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

MODIFIED 10/96

PSJN2/179013.1

9855-7U1

COMPOSITIONS AND METHODS FOR EFFECTING AND REGULATING ADP-ACTIVATION OF DNA MISMATCH BINDING PROTEINS

GOVERNMENT SUPPORT

This research was supported in part by U.S. Government funds (NIH grants numbers CA56542 and CA67007 and NRSA grant CA73134), and the U.S. Government may therefore have certain rights in the invention.

FIELD OF THE INVENTION

The field of the invention is DNA mismatch protein binding, including animal models for tumorigenesis, apoptosis, and aging.

BACKGROUND OF THE INVENTION

The development of transgenic animals and nullizygous animal models has provided important new avenues for the study of specific gene functions in differentiation, embryogenesis and neoplastic development (Palmiter et al., 1986, Ann. Rev. Genet. 20:465-499). Transgenic animals frequently serve as model systems for the study of various disease states and also provide an experimental system in which to test compounds for their ability to regulate disease. Nullizygous animals are similarly useful as experimental systems for the testing of compounds useful for diagnosis, treatment, or both, of disease.

Lukkarinen et al. (1997, Stroke 28:639-645) teaches that gene constructs which enable the generation of transgenic mice also enable the generation of other transgenic rodents, including rats. Similarly, nullizygous mutations in a genetic locus of an animal of one species can be replicated in an animal of another species having a genetic locus highly homologous to the first species. For example, many genetic loci

are highly homologous among mammals, and even more highly homologous among subgroups of mammals, such as among rodents.

The mutator hypothesis of tumorigenesis suggests that loss in an organism of a chromosomal stability function, a chromosomal maintenance function, or both, results in an elevated mutation rate in the organism. An elevated mutation rate hastens accumulation of the numerous mutations required for multistep carcinogenesis (Loeb, 1991, Cancer Res 51:3075-3079).

Loss of the function of p53 protein has been proposed to increase cellular hypermutability in an organism, thereby accelerating tumorigenesis, although a clear role for p53 protein in genomic instability remains controversial (Kastan et al., 1992, Cell 71:587-597; Fishel et al., 1997, Curr. Opin. Genet. Dev. 7:105-113). *p53*, the gene encoding p53 protein, is frequently mutated in a wide range of human cancers including, but not limited to, colonic tumors (Fearon et al., 1990, Cell 61:759-767). Transgenic mice nullizygous for the *p53* gene are viable and susceptible to tumorigenesis (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nat. Genet. 11:64-70; Donehower et al., 1992, Nature 356:215-221; Jacks et al., 1994, Curr. Biol. 4:1-7; Purdie et al., 1994, Oncogene 9:603-609).

Although nullizygous *p53* mice can be used as models of carcinogenesis, the rates at which such mice develop tumors can be slower than what is desirable, particularly for large-scale screening studies involving numerous potential anti-cancer therapeutic or prophylactic compositions. What is needed is a transgenic mouse which, when exposed to a carcinogen, succumbs to tumorigenesis caused by the carcinogen more readily than does a nullizygous *p53* mouse and which, even when not exposed to an identifiable carcinogen, succumbs to tumors more readily than does a nullizygous *p53* mouse .

Critical unmet needs also exist for animal models of programmed cell death (apoptosis) and of aging.

In addition, there is a critical unmet need for the identification and isolation of MutL homolog derivatives which affect the rate of DNA mismatch repair

and for identification of compounds capable of affecting the ability of MutL homologs to influence the activity of MutS homologs and other components of DNA mismatch repair pathways. The present invention satisfies these needs.

SUMMARY OF THE INVENTION

The invention relates to a method of influencing the activity of a MutS homolog. The method comprises contacting the MutS homolog with a MutL homolog derivative in the presence of a solution comprising duplex DNA and ADP. The MutL homolog derivative interacts with the MutS homolog and influences the ability of the MutS homolog to bind with a mismatched region of the duplex DNA.

In a preferred embodiment, the MutS homolog is selected from the group consisting of the *E. coli* MutS protein, an hMSH2 protein dimer, a complex comprising an hMSH2 protein molecule and either an hMSH3 protein molecule or an hMSH6 protein molecule, a yeast MSH2 protein dimer, a complex comprising a yeast MSH2 protein molecule and either a yeast MSH3 protein molecule or a yeast MSH6 protein molecule, a dimer of a rat homolog of hMSH2, a dimer of a *Xenopus* homolog of hMSH2, a dimer of a *Drosophila* homolog of hMSH2, a dimer of a murine homolog of hMSH2, and a complex comprising a murine homolog of hMSH2 and either a murine homolog of hMSH3 or a murine homolog of hMSH6. More preferably, the homolog comprises a complex comprising an hMSH2 protein molecule and an hMSH6 protein molecule.

The invention also relates to a method of identifying a MutL homolog derivative which is capable of influencing the activity of a MutS homolog. The method comprises contacting the MutS homolog with a fluid comprising duplex DNA and ADP in the presence of either the MutL homolog derivative or a naturally occurring MutL homolog. A difference between the ability of the MutS homolog to bind with a mismatched region of the duplex DNA in the presence of the MutL homolog derivative and the ability of the MutS homolog to bind with a mismatched region of the duplex

DNA in the presence of the naturally occurring MutL homolog is an indication that the MutL homolog derivative is capable of influencing the activity of a MutS homolog.

In the remainder of this Summary of the Invention section, "MutS homolog" should be construed to include any one or more of a MutS homolog, a MutS homolog in combination with a MutL homolog, and a MutS homolog in combination with a MutL homolog derivative.

The invention also relates to a method of binding a MutS homolog to a mismatched region of a duplex DNA molecule, the method comprising contacting the homolog with the duplex DNA molecule in the presence of a solution comprising an ADP molecule, whereby the homolog binds to the mismatched region. The MutS homolog may, for example, be one of those described above.

In other preferred embodiments, the solution comprises at least about 20 micromolar ADP and the solution comprises less than about 5 micromolar ATP.

In another preferred embodiment, the solution further comprises ATP and the ratio of the concentration of ADP in the solution to the ratio of the concentration of ATP in the solution is greater than about two.

Also preferably, the solution is substantially free of ATP.

In yet other preferred embodiments, the duplex DNA molecule is suspended in the solution and the homolog is suspended in the solution.

The invention also relates to a method of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules. The method comprises providing a MutS homolog and a solution comprising an ADP molecule and the population of DNA molecules, whereby the homolog binds to the mismatched region, and segregating the homolog from the population, whereby the duplex DNA molecule is segregated from the population.

In a preferred embodiment, the homolog is bound to a solid support and the process of segregating the homolog from the population comprises rinsing the solid support with a solution which does not comprise the population.

In another preferred embodiment, the population comprises a plurality of cDNA molecules, wherein each of the cDNA molecules is made by reverse transcription of an RNA molecule obtained from an organism.

In yet another preferred embodiment, the organism is a mammal, preferably, a human.

In another preferred embodiment, the method further comprises contacting the homolog with a solution comprising an ATP molecule after segregating the homolog from the population, whereby the mismatched region dissociates from the homolog.

There is also included in the invention a method of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence. The method comprises annealing a first single-stranded DNA molecule and a second single-stranded DNA molecule to form a duplex DNA molecule, wherein the first molecule has the sample sequence and wherein the second molecule has a nucleotide sequence which is complementary to the reference sequence, whereby if there is a difference between the sample sequence and the reference sequence then the duplex DNA molecule has a mismatched region, thereafter contacting the duplex DNA molecule and a MutS homolog in the presence of a solution comprising an ADP molecule, whereby the homolog binds specifically to the duplex DNA molecule if the duplex DNA molecule comprises the mismatched region, and thereafter determining whether the homolog binds specifically to the duplex DNA molecule, whereby specific binding of the homolog to the duplex DNA molecule is an indication that there is a difference between the sample nucleotide sequence and the reference nucleotide sequence.

In a preferred embodiment of this aspect of the invention, the sample nucleotide sequence comprises the sequence of a region of a gene obtained from a first organism and the reference nucleotide sequence comprises the sequence of the region obtained from a second organism.

In other preferred embodiments, the first organism and the second organism are the same species, preferably, a human.

In yet another preferred embodiment, the sample nucleotide sequence comprises the sequence of a region of a gene obtained from an organism and the reference nucleotide sequence comprises a consensus nucleotide sequence of the region. Preferably, the gene is the human *msh2* gene.

Also included in the invention is a kit for separating a mismatched duplex DNA molecule from a population of molecules, the kit comprising a MutS homolog, a linker for binding the homolog to a solid support, and an ADP molecule.

There is also provided in the invention a method of detecting a predisposition of a mammal to carcinogenesis. The method comprises annealing a first single-stranded DNA molecule and a second single-stranded DNA molecule to form a duplex DNA molecule, wherein the first molecule has the nucleotide sequence of a region of a mammalian gene associated with carcinogenesis and wherein the second molecule has a nucleotide sequence which is complementary to the consensus nucleotide sequence of the region, whereby if there is a sequence difference between the first molecule and the second molecule then the duplex DNA molecule has at least one mismatched region, thereafter contacting the duplex DNA molecule and a MutS homolog in the presence of a solution comprising an ADP molecule, whereby the homolog binds to the duplex DNA molecule if the duplex DNA molecule comprises a mismatched region, and thereafter determining whether the homolog binds to the duplex DNA molecule, whereby binding of the homolog to the duplex DNA molecule is an indication of the predisposition of the mammal to carcinogenesis.

In a preferred embodiment of this aspect of the invention, the mammalian gene associated with carcinogenesis is selected from the group consisting of the *msh2* gene, the *msh3* gene, the *msh6* gene, the *mlh1* gene, the *pms2* gene, the *brca1* gene, the *brca2* gene, the *pten* gene, and the *p53* gene. Preferably, the mammalian gene is the human *msh2* gene and the predisposition of a mammal for

carcinogenesis is predisposition of a human for hereditary non-polyposis colorectal cancer.

There is also provided a method of fractionating a population of duplex DNA molecules. The method comprises contacting the population with a MutS homolog in the presence of ADP, whereby the homolog is capable of binding to a duplex DNA molecule of the population if the duplex DNA molecule comprises a mismatched region, segregating the homolog from the population, and contacting the homolog with a dissociation solution comprising an ATP molecule, whereby the duplex DNA molecule dissociates from the homolog, whereby the population is fractionated.

In addition, there is provided a method of selectively amplifying at least one mismatched duplex DNA molecule of a population of duplex DNA molecules. The method comprises contacting the population with a MutS homolog in the presence of a solution comprising an ADP molecule, whereby the homolog binds to the at least one mismatched duplex DNA molecule, thereafter segregating the homolog from the population, whereby each the at least one mismatched duplex DNA molecule is segregated from the population, and thereafter amplifying each the at least one mismatched duplex DNA molecule using a PCR technique, whereby each of the at least one mismatched duplex DNA molecule is selectively amplified.

Also included is a method of detecting the presence of a genetic polymorphism in the genome of an animal. The method comprises amplifying a region of each of two copies of a gene of the animal to yield an amplified first copy and an amplified second copy, thereafter mixing and denaturing each of the first copy and the second copy to yield a first mixture of nucleic acids comprising a first sense copy, a first antisense copy, a second sense copy, and a second antisense copy, thereafter annealing the nucleic acids in the first mixture to yield a second mixture of nucleic acids comprising the first copy, the second copy, a first sense-second antisense duplex DNA molecule, and a second sense-first antisense duplex DNA molecule, whereby if the first sense copy and the second antisense copy are not completely complementary

then the first sense-second antisense duplex DNA molecule will have a first mismatched region, and whereby if the second sense copy and the first antisense copy are not completely complementary then the second sense-first antisense duplex DNA molecule will have a second mismatched region, thereafter contacting the second mixture with a MutS homolog in the presence of a solution comprising an ADP molecule, whereby the homolog is capable of binding to either of the first mismatched region or the second mismatched region, thereafter segregating the homolog from the second mixture, and thereafter detecting the presence or absence of either of the first sense-second antisense duplex DNA molecule or the second sense-first antisense duplex DNA molecule, whereby the presence of either of the first sense-second antisense duplex DNA molecule or the second sense-first antisense duplex DNA molecule is an indication that the animal has a genetic polymorphism.

The invention further includes a composition for segregating a mismatched duplex DNA molecule from a population of molecules. The composition comprises a MutS homolog bound to a solid support, wherein the support is in liquid contact with a solution comprising an ADP molecule.

Further included is a kit for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence. The kit comprises a pair of primers adjacent the region for amplifying the region, a duplex DNA molecule having the reference nucleotide sequence, a solid support, a MutS homolog, a linker for binding the MutS homolog to the solid support, and an ADP molecule.

In addition, the invention includes a nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene. Preferably, the mammal is a rodent; more preferably, a mouse.

In preferred embodiments, the mammal has an average survival time shorter than the average survival time of a nullizygous *Msh2* mammal of the same species and the mammal has an average survival time shorter than the average survival time of a nullizygous *p53* mammal of the same species. In addition, the mammal exhibits female-specific embryonic lethality.

There is also included a method of making a nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene. The method comprises mating a first mammal of a species, the first mammal comprising at least one null allele of the *Msh2* gene of the species with a second mammal of the species, the second mammal comprising at least one null allele of the *p53* gene of the species.

In addition, there is provided a method of identifying a compound which affects tumorigenesis in a mammal comprising administering the compound to a first nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene, and comparing tumor incidence in the first transgenic mammal with tumor incidence in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered the compound, wherein a difference in tumor incidence in the first transgenic mammal compared with tumor incidence in the second transgenic mammal is an indication that the compound affects tumorigenesis in the mammal.

Further included is a method of identifying a compound which affects apoptosis in a mammal comprising administering the compound *in utero* to a first nonhuman mammal embryo which is nullizygous for both the *Msh2* gene and the *p53* gene, and comparing embryonic development in the first transgenic mammal embryo with embryonic development in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered the compound, wherein a difference in embryonic development of the first transgenic mammal compared with embryonic development of the second transgenic mammal is an indication that the compound affects apoptosis in the mammal.

The invention also includes a method of identifying a compound which affects the aging process in a mammal comprising administering the compound *in utero* to a first nonhuman mammal embryo which is nullizygous for both the *Msh2* gene and the *p53* gene, and comparing embryonic development in the first transgenic mammal embryo with embryonic development in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered

the compound, wherein a difference in embryonic development of the first transgenic mammal compared with embryonic development of the second transgenic mammal is an indication that the compound affects the aging process in the mammal.

In addition, there is provided a cell line which is nullizygous for both the *Msh2* gene and the *p53* gene, wherein the cell line is made by culturing a cell obtained from a nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an image which depicts proteins detected in preparations containing the hMSH2/hMSH6 complex. Proteins were separated by SDS-PAGE. Molecular weight markers are shown on the left of the Figure and in the lane designated "MWM." Lane 1 depicts proteins present in a crude extract which was obtained from the insect cells described herein. Lane 2 depicts proteins present in peak fractions obtained from the nickel affinity column purification step described herein. Lane 3 depicts proteins present in peak fractions obtained from the PBE anion exchange column purification step described herein. Proteins were stained using Coomassie Brilliant blue dye. The positions of protein bands corresponding to hMSH2 and to hMSH6 are indicated.

Figure 2, comprising Figures 2A, 2B, 2C, 2D, 2E, and 2F, depict binding of hMSH2/hMSH6 protein complex to mismatched and non-mismatched DNA. Figure 2A is an image of the results of a gel mobility shift assay performed using the G/T-mismatched 81-base pair DNA substrate described herein. The concentrations of complex are indicated along the top of the image. The position of the S-shifted electrophoretic band is indicated by "S". Figure 2E is a graph which depicts the relationship between the concentration of complex and the amount of product corresponding to the S-shifted electrophoretic band in Figure 2A, as assessed using a phosphoimaging device. Figure 2B is an image of the results of a gel mobility shift assay performed using the homologous 81-base pair DNA substrate described herein.

The concentrations of complex are indicated along the top of the image. The position of the NS-shifted electrophoretic band is indicated by "NS". Figure 2F is a graph which depicts the relationship between the concentration of complex and the amount of product corresponding to the NS-shifted electrophoretic band in Figure 2B, as assessed using a phosphoimaging device. Figure 2C is an image which depicts the results of a DNase footprint assay performed using the G/T-mismatched 81-base pair DNA substrate described herein. The concentrations of complex are indicated along the top of the image. The position of the G residue of the G/T-mismatched substrate is indicated by "G", and the approximate region of the substrate protected from DNase cleavage by the complex is indicated by a vertical line. Figure 2D is an image which depicts the results of a DNase footprint assay performed using the homologous 81-base pair DNA substrate described herein. The concentrations of complex are indicated along the top of the image. The position of the G/C base pair corresponding to the G/T-mismatched base pair of the mismatched substrate is indicated by "G".

Figure 3, comprising Figures 3A, 3B, and 3C, depicts kinetic characterization data for the ATPase activity of the hMSH2/hMSH6 protein complex. Figure 3A is a graph which depicts the extent of ATP hydrolysis after 30 minutes as a function of selected concentrations of the complex, as assessed in the presence or absence of mismatched DNA, in the presence or absence of homologous DNA, and in the presence of magnesium chloride. Figure 3B is an image which depicts the results of TLC analysis of the products of the ATPase activity of the complex in the presence of mismatched DNA. Lane 1 depicts the products formed in the ATPase reaction mixture in the absence of the complex. Lane 2 depicts the products formed in the ATPase reaction mixture in the presence of 60 nanomolar complex. The positions of AMP, ADP, and ATP are indicated to the right of the lane. Figure 3C is a double-reciprocal plot of steady-state ATPase activity at selected ATP concentrations, as assessed in the presence or absence of homologous DNA and in the presence or absence of mismatched DNA. The inset depicts a magnification of the portion of the plot near the intercepts of the 1/V plots with the horizontal and vertical axes.

Figure 4, comprising Figures 4A, 4B, 4C, and 4D, depicts the results of gel mobility shift assays used to assess the ability of various adenine nucleotides to convert the S-shifted electrophoretic band to the NS-shifted electrophoretic band. Figure 4A is an image of an assay in which the product corresponding to the S-shifted electrophoretic band was incubated in the presence of ATP at the concentration listed along the top of the image. Figure 4B is an image of an assay in which the product corresponding to the S-shifted electrophoretic band was incubated in the presence of adenosine-5'-O-3'-thiotriphosphate (ATP-γ-S) at the concentration listed along the top of the image. Figure 4C is an image of an assay in which the product corresponding to the S-shifted electrophoretic band was incubated in the presence of ADP at the concentration listed along the top of the image. In Figures 4A, 4B, and 4C, "-" indicates that no complex was included in the assay mixture. Figure 4D is a graph which depicts quantitated results obtained using the results depicted in Figures 4A, 4B, and 4C, as assessed using a phosphoimaging device.

Figure 5 is a bar graph which depicts the effect of selected nucleotides, deoxynucleotides, and nucleotide analogs on G/T mismatch binding by the complex, relative to the degree of binding observed in the absence of a (deoxy)nucleotide or analog. The effect of each indicated (deoxy)nucleotide or analog was assessed at 25 micromolar (left bar of each pair) and at 250 micromolar (right bar of each pair).

Figure 6, comprising Figures 6A, 6B, 6C, 6D, and 6E, depicts the effects of ATP hydrolysis or ADP binding by the hMSH2/hMSH6 protein complex on mismatched DNA binding. Figure 6A is an image which depicts the results of gel mobility shift assays performed in the presence or absence of 15 micromolar ATP. Magnesium chloride was added at the time designated "0", and samples of the assay mixture were collected at the indicated times (in minutes). The binding reaction in each mixture was halted by the addition of 5 millimolar EDTA. Figure 6B is an image which depicts the results of gel mobility shift assays performed in the presence or absence of 15 micromolar ATP-γ-S. Magnesium chloride was added at the time designated "0", and samples of the assay mixture were collected at the indicated times

(in minutes). The binding reaction in each mixture was halted by addition of 5 millimolar EDTA. Figure 6C is a graph of the results depicted in Figures 6A and 6B, as assessed using a phosphoimaging device. Figure 6D is an image which depicts the results of gel mobility shift assays performed in the presence of the indicated (in millimolar) concentrations of ATP or ADP or both. In each of Figures 6A, 6B, and 6D, the left-most lane depicts results obtained using an assay mixture which did not contain the complex, and S-shifted and NS-shifted electrophoretic bands are indicated by "S" and "NS", respectively. Figure 6E is a graph of the results depicted in Figure 6D, as assessed using a phosphoimaging device.

Figure 7 comprises Figures 7A, 7B, 7C, and 7D. Figure A is a diagram which depicts the mechanism by which the hMSH2/hMSH6 complex is switched to the "ON" form, which is capable of binding mismatched DNA, from the "OFF" form, which has a much lower affinity for mismatched DNA. Switching from the "OFF" to the "ON" form involves hydrolysis of ATP bound to the complex, which hydrolysis is catalyzed by the complex. Switching from the "ON" to the "OFF" form involves displacement of ADP bound to the complex by an ATP molecule. Figure 7B is a graph which depicts the results obtained in the assays described herein for detecting the rate of a single round of ATP hydrolysis by the complex. Figure 7C is a graph which depicts the results obtained in assays described herein for detecting the rate of a single round of ATP hydrolysis by the complex in the presence of selected amounts of mismatched DNA. Figure 7D is a graph which depicts the results obtained in assays described herein for detecting the exchange of ADP for ATP in the presence or absence of mismatched DNA. "%ADP bound" means the percentage of ADP that was initially bound to the complex which remained bound at the time indicated in the graph.

Figure 8, comprising Figures 8A, 8B, 8C, and 8D, depicts the results of experiments performed to assess the effects of ATP, homologous DNA, or both, on the dissociation of the hMSH2/hMSH6 complex from DNA. Figure 8A is an image of the results obtained from gel mobility shift assays in which complex-bound mismatched DNA was incubated with ATP for the time indicated in the image. Figure 8B is an

image of the results obtained from gel mobility shift assays in which complex-bound mismatched DNA was incubated with ATP and a 400-fold excess of homologous DNA for the time indicated in the image. Figure 8C is an image of the results obtained from gel mobility shift assays in which complex-bound mismatched DNA was incubated with a 400-fold excess of homologous DNA for the time indicated in the image. Figure 8D is an image of the results obtained from gel mobility shift assays in which the complex was incubated with homoduplex DNA probe for fifteen minutes at 37°C (Lane A), the assay mixture was cooled to 4°C, and a 1,100-fold excess of unlabeled competitor homoduplex DNA was added (Lane B). In each of Figure 8A, 8B, 8C, and 8D, "-" indicates assay mixtures which did not comprise the complex.

Figure 9 is a diagram which depicts the model of the hMSH2/hMSH6 protein complex binding to mismatched DNA described herein. The ADP-bound form of the complex, which is shown in the center of the diagram, is competent to bind mismatched DNA, as shown at the bottom of the diagram. Mismatched DNA-bound complex may be displaced therefrom by displacing the ADP molecule bound to the complex with an ATP molecule, which yields the ATP-bound form of the complex, which is shown at the top of the diagram. The ATP-bound form of the complex may be converted to the ADP-bound form by hydrolysis of the complex-bound ATP molecule, catalyzed by ATPase activity of the complex.

Figure 10 lists the nucleotide sequence of single nucleotide chains of the 39- and 81-base pair DNA substrates described herein (SEQ ID NOS:1-6).

Figure 11, comprising Panels A, B, C, and D, is a series of images, each of which depicts a whole mount view of an *Msh2*$^{-/-}$*p53*$^{-/-}$ embryo at day 11.5 of development. The embryo depicted in Panel A is a male *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryo, and exhibits phenotypically normal embryonic development, relative to mice having the same genotypic background. The embryos depicted in Panels B, C, and D are female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos that are littermates of the male mouse depicted in Panel A. The female mouse embryos depicted in Panels B, C, and D exhibit

developmental arrest having a phenotype corresponding to that expected at day 9.5 of embryonic development.

Figure 12, comprising Panels A, B, C, D, E, and F, is a series of images, each of which depicts a paraffin embedded section obtained from an 11.5 day old female mouse embryo. The images in Panels A, C, and E each depict a section obtained from an 11.5 day old normal embryo. The images in Panels B, D, and F each depict a section obtained from an 11.5 day old *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryo. The sections depicted in Panels A and B are at 100× magnification and are stained with hematoxylin and eosin. Magnification of the normal embryo is of the somite region of a sagittal section. The sections depicted in Panels C and D are at 100× magnification and are chromogenically-TUNEL stained. The sections depicted Panels E and F are at 40× magnification and are fluorescently-TUNEL stained. Cells undergoing apoptosis in normal female embryos were rare; chromogenically- and fluorescently-TUNEL stained cells depicted in Panels C and E represent circumscribed apoptotic foci normally found in developing mouse embryos.

Figure 13 is a graph which depicts Kaplan-Meier survival probabilities of *Msh2*$^{-/-}$, *p53*$^{-/-}$, and *Msh2*$^{-/-}$*p53*$^{-/-}$ mice.

Figure 14 is an image of a polyacrylamide gel which was used to separate amplification transcript-length polymorphs resulting from microsatellite instability at the *D17Mit123* (CA_{26}) locus in *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. Samples were obtained from normal tissue and from thymic lymphoma tissue from each of five *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. The five individual mice are identified as m87, m132, m148, m149, and m205. Allele length alterations generated by microsatellite instability in the tumor are indicated by arrows.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to a method of influencing the actvity of a MutS homolog. The method comprises contacting the MutS homolog with a MutL homolog derivative in the presence of a solution comprising duplex DNA and ADP. The MutL

homolog derivative interacts with the MutS homolog and influences the ability of the MutS homolog to bind with a mismatched region of the duplex DNA.

As used herein, a "MutL homolog derivative" means a molecule having significant structural similarity to a naturally occurring MutL homolog or to a portion of such a MutL homolog. MutL homolog derivatives include, for example, naturally occurring MutL homologs and polypeptides having an amino acid sequence at least 50%, preferably at least 75%, more preferably at least 90%, still more preferably at least 95%, and most preferably completely identical to all or a portion of the amino acid sequence (e.g. from about five amino acid residues to all amino acid residues) of a naturally occurring MutL homolog. MutL homolog derivatives also include, for example, chemical derivatives of naturally occurring MutL homologs and chemical derivatives of the polypeptides described in the preceding sentence. MutL homolog derivatives may have one or more modified amino acid residues, conservative replacement(s) of one or more amino acid residue, other modifications known in the art for making physiologically acceptable peptides and peptidomimetics, or some combination of these. Naturally-occurring MutL homologs include, but are not limited to, the MutL homologs present in publicly available databases (e.g. yeast MutL homologs MLH1, MLH2, PMS1 and human MutL homologs hMLH1, hPMS1, and hPMS2).

In a preferred embodiment, the MutS homolog is selected from the group consisting of the *E. coli* MutS protein, an hMSH2 protein dimer, a complex comprising an hMSH2 protein molecule and either an hMSH3 protein molecule or an hMSH6 protein molecule, a yeast MSH2 protein dimer, a complex comprising a yeast MSH2 protein molecule and either a yeast MSH3 protein molecule or a yeast MSH6 protein molecule, a dimer of a rat homolog of hMSH2, a dimer of a *Xenopus* homolog of hMSH2, a dimer of a *Drosophila* homolog of hMSH2, a dimer of a murine homolog of hMSH2, and a complex comprising a murine homolog of hMSH2 and either a murine homolog of hMSH3 or a murine homolog of hMSH6. More preferably, the

homolog comprises a complex comprising an hMSH2 protein molecule and an hMSH6 protein molecule.

The invention also relates to a method of identifying a MutL homolog derivative which is capable of influencing the actvity of a MutS homolog. The method comprises contacting the MutS homolog with a fluid comprising duplex DNA and ADP in the presence of either the MutL homolog derivative or a naturally occurring MutL homolog. A difference between the ability of the MutS homolog to bind with a mismatched region of the duplex DNA in the presence of the MutL homolog derivative and the ability of the MutS homolog to bind with a mismatched region of the duplex DNA in the presence of the naturally occurring MutL homolog is an indication that the MutL homolog derivative is capable of influencing the actvity of a MutS homolog.

In the remainder of this application, "MutS homolog" should be construed to include any one or more of a MutS homolog, a MutS homolog in combination with a MutL homolog, and a MutS homolog in combination with a MutL homolog derivative.

The present invention also provides a method of inducing stable association of a MutS homolog with a mismatched region of a DNA molecule. The methods of the invention are based on the discovery that binding of an ADP molecule to a MutS homolog stabilizes the association of the ADP-bound homolog with a mismatched region of a DNA molecule.

Homologs of the *E. coli* MutS protein are known to be involved in DNA mismatch repair, including the MutS homologs encoded by the human *hMSH2*, *hMSH3*, and *hMSH6* genes, designated hMSH2, hMSH3, and hMSH6, respectively. hMSH2 is capable of forming a protein complex with either hMSH3 or hMSH6 and these two complexes, designated the hMSH2/hMSH3 protein complex and the hMSH2/hMSH6 protein complex, respectively, are capable of binding to mismatched DNA. A MutS homolog can be caused to dissociate from a mismatched region of a DNA molecule by contacting the homolog with ATP. It is believed that contacting the homolog with ATP caused an ATP-hydrolysis-dependent translocation of the homolog

along the mismatched DNA strand. However, in order that the association of a MutS homolog with a mismatched region of a DNA molecule be exploited as a useful phenomenon, it is necessary that the complex which is formed is stabilized. According to the discovery of the present invention, a means of stabilizing a MutS homolog/DNA complex is provided. The ability to provide a stable MutS homolog/DNA complex facilitates the exploitation of the formation of this complex as a useful entity as described herein.

The methods of the invention include a method of binding a MutS homolog to a mismatched region of a duplex DNA molecule, comprising contacting the MutS homolog and the duplex DNA molecule in the presence of a solution comprising an ADP molecule, whereby the MutS homolog binds to the mismatched region.

In the method of the invention of binding a MutS homolog to a mismatched region of a duplex DNA molecule, the MutS homolog may be any MutS homolog which is presently known to be or is discovered to be involved in DNA mismatch repair. Thus, by way of example, the MutS homolog useful in the methods, products, and compositions of the invention may be the *E. coli* MutS protein, an hMSH2 protein dimer, a complex comprising an hMSH2 protein molecule and either an hMSH3 protein molecule or an hMSH6 protein molecule, a yeast MSH2 protein dimer, a complex comprising a yeast MSH2 protein molecule and either a yeast MSH3 protein molecule or a yeast MSH6 protein molecule, a dimer of a rat homolog of hMSH2 (e.g. GenBank accession number X93591), a dimer of a *Xenopus* homolog of hMSH2 (Varlet et al., 1994, Nucl. Acids Res. 22:5723-5728), a dimer of a *Drosophila* homolog of hMSH2 (e.g. GenBank accession number U17893), a dimer of a murine homolog of hMSH2 (e.g. GenBank accession number X93591, Varlet et al., 1994, Nucl. Acids Res. 22:5723-5728), a complex comprising a murine homolog of hMSH2 and either a murine homolog of hMSH3 (e.g. Rep-3; Linton et al., 1989, Mol. Cell. Biol. 9:3058-3072; Smith et al., 1990, Mol. Cell. Biol. 10:6003-6012) or a murine homolog of hMSH6 (e.g. Gen Bank accession number U42190), and the like. It is understood that, given the high degree of similarity among mammalian MutS homologs

(Fishel et al., 1997, Curr. Op. Genet. Develop. 7:105-113), a dimer of any mammalian hMSH2 homolog can be used in the methods of the invention. Similarly, any complex comprising any mammalian hMSH2 homolog and either any mammalian hMSH3 homolog or any mammalian hMSH6 homolog can be used in the methods of the invention.

In the method of the invention of binding a MutS homolog to a mismatched region of a duplex DNA molecule, the DNA molecule may be any duplex DNA molecule having a mismatched region. By way of example, the DNA molecule may be a linear DNA molecule, a circularized DNA molecule such as a plasmid or a viral genome, a chromosome, a cDNA generated by reverse transcription of an RNA molecule, a PCR primer, a PCR product, a complex formed between a single-stranded DNA probe and another single-stranded DNA molecule, and the like. The mismatched region may be any region of a duplex DNA molecule in which the two DNA strands of the molecule are not completely complementary. By way of example, the mismatched region may comprise one or more pairs of mismatched nucleotides in an otherwise complementary region of a duplex DNA molecule, a region of a duplex DNA molecule wherein a thymine dimer exists on one DNA strand of the molecule, a region of a duplex DNA molecule comprising a nucleotide which has been covalently modified by an agent capable of reacting with a nucleotide, such as cisplatin, a region of a duplex DNA molecule which comprises an alkyl-O-6-methyl guanine residue, a region of a duplex DNA molecule which comprises a single stranded loop of one or more nucleotides, a region of a duplex DNA molecule which comprises a pyrimidine dimer, and the like.

While any amount of ADP can be used in the method of the invention of binding a MutS homolog to a mismatched region of a duplex DNA molecule, it is preferred that the homolog be contacted with the DNA molecule in the presence of a solution comprising at least about 20 micromolar ADP. As described with greater particularity in Example 1, ATP displaces ADP from the MutS homolog. Thus, it is important either that the concentration of ATP in the solution be minimized, for

example by maintaining the concentration of ATP lower than about 5 micromolar, or that the ratio of the concentration of ADP in the solution to the concentration of ATP in the solution be greater than a minimum value, such as about two. Preferably, the solution is substantially free of ATP, by which is meant that the concentration of ATP in the solution is lower than 5 micromolar; preferably, the concentration of ATP in the solution is 1 micromolar or lower.

In the method of the invention of binding a MutS homolog to a mismatched region of a duplex DNA molecule, either or both of the MutS homolog and the DNA molecule may be suspended in the ADP-containing solution. Alternately, either or both of the MutS homolog or the DNA molecule may be fixed to a surface which contacts the ADP-containing solution. Where the MutS homolog is fixed to a first surface, such as a latex bead or the like, and the DNA molecule is fixed to a second surface, such as a glass slide, a microwell plate, or the like, it is necessary that the first and second surfaces be moveable with respect to one another, such that the MutS homolog and the DNA molecule are capable of contacting one another in the presence of the ADP-containing solution.

The MutS homolog may be bound to a surface using any known method for attaching a protein to a surface. For example the MutS homolog may be bound to a surface by way of an antibody which is covalently bound to the surface and which has a variable region which specifically binds to the MutS homolog. By way of example, an antibody which specifically binds to hMSH2 such as the antibody described by Kinzler et al. (PCT application WO96/41192, published December 19, 1996) may be used to bind an hMSH2 protein dimer or a complex comprising an hMSH2 protein molecule and either an hMSH3 protein molecule or an hMSH6 protein molecule to a surface to which the antibody is fixed. Methods of fixing an antibody to a surface have been described in the art (e.g. Harlow et al., 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York).

The duplex DNA molecule may be bound to a surface using any known method for attaching a nucleic acid to a surface. By way of example, the nucleic acid

may be covalently linked to a biotin molecule and the surface may be linked to or coated with a streptavidin molecule, whereby the streptavidin molecule is capable of binding the biotin molecule, thereby linking the nucleic acid to the surface.

The methods of the invention also include a method of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules. This method comprises contacting a MutS homolog and the population of DNA molecules in the presence of a solution comprising an ADP molecule and then segregating the homolog from the population. As described herein, the MutS homolog is capable of binding to the mismatched region of the DNA molecule, whereupon the DNA molecule may be physically manipulated by manipulating the MutS homolog or a solid support linked to the homolog.

The method used to segregate the MutS homolog from the population of DNA molecules in the method may be any method by which the concentration of the homolog in at least a portion of the homolog-population mixture can be changed relative to the concentration of the population. Such methods include, but are not limited to, linking the homolog to a surface, such as a polystyrene surface, which contacts the mixture, precipitating the homolog from the mixture, precipitating DNA molecules which are not bound to the homolog from the mixture, linking each DNA molecule of the population to a surface which contacts the mixture, and hydrolyzing or otherwise degrading the DNA molecules of the population after contacting the homolog with the population.

By way of example, the method of the invention of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules may be performed by binding a MutS homolog to a solid support, contacting the solid support with the population of DNA molecules, and rinsing the solid support with a solution which does not comprise the population of DNA molecules. In this example, a mismatched DNA molecule in the population of DNA molecules binds to the MutS homolog and thereby becomes linked to the solid support, and the mismatched DNA molecule is segregated from the other DNA molecules of the population by rinsing the

support with a solution which carries the other DNA molecules away from the solid support. Thus, according to this example, the mismatched DNA molecule is physically separated from the other DNA molecules of the population.

It is not necessary that the just-described method result in separation of the DNA molecule having a mismatched region from the population given that the molecule and the population are contained in different containers at the conclusion of the method. By way of example, it is sufficient in the optical affinity biosensor system (OABS) described elsewhere herein that a DNA molecule comprising a mismatched region associate with the detector surface of the OABS and that non-mismatched DNA molecules do not associate with the detector surface of the OABS. Thus, for example, in OABS methods for detection of mismatched DNA molecules, a MutS homolog may be associated with the detector surface of the OABS, whereby a DNA molecule having a mismatched region binds to the homolog in the presence of ADP and is detected, and whereby a DNA molecule not having a mismatched region does not bind appreciably to the homolog and is not detected.

The DNA molecules of the population of DNA molecules of the method of the invention of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules may be any DNA molecules, so long as at least one of the DNA molecules is a duplex DNA molecule having a mismatched region. By way of example, each of the DNA molecules of the population may be a cDNA molecule generated by reverse transcription of an RNA molecule obtained from an organism such as an animal, a mammal, or a human.

In a variation of the method of the invention of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules, the method further comprises contacting the MutS homolog with a solution comprising an ATP molecule after segregating the homolog from the population of DNA molecules, whereby the mismatched DNA molecule bound to the homolog dissociates therefrom. According to this variation, the method can be used as a method for purifying a mismatched DNA molecule from a population of DNA molecules which includes both

mismatched and non-mismatched DNA molecules. By way of example, the population of DNA molecules may be made by annealing one or more single-stranded DNA molecules each having the consensus nucleotide sequence of a gene with single-stranded DNA molecules made by reverse transcription of mRNA obtained from an organism such as a mammal, whereby duplex DNA molecules not having a mismatched region are formed between single-stranded DNA molecules which are complementary to one another, and duplex DNA molecules having a mismatched region are formed between single-stranded DNA molecules which are complementary in some regions, but are not complementary in at least one region bounded by complementary regions. Thus, the method of the invention of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules may be used to detect expression of a gene having a nucleotide sequence which differs from the consensus sequence of the gene in an organism.

The methods of the invention also include a method of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence. To perform this method, a first single-stranded DNA molecule having the sample nucleotide sequence is annealed with a second single-stranded DNA molecule having a nucleotide sequence which is completely complementary to the reference sequence to form a duplex DNA molecule. If there is a difference between the sample sequence and the reference sequence then the duplex DNA molecule has at least one mismatched region. According to the method, after forming the duplex DNA molecule, the duplex DNA molecule is contacted with a MutS homolog in the presence of a solution comprising an ADP molecule. Thus, if the duplex DNA molecule has a mismatched region, the homolog will bind to it. Next, according to the method, it is determined whether the homolog binds specifically to the duplex DNA molecule. Specific binding of the homolog to the duplex DNA molecule is an indication that there is a difference between the sample nucleotide sequence and the reference nucleotide sequence.

In the method of the invention of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence, any method of

determining whether the homolog binds specifically to the duplex DNA molecule may be used. By way of example, the gel mobility shift assay or the DNase footprint assay described herein in Example 1 may be used to detect specific binding of the MutS homolog to the duplex DNA molecule. Also by way of example, after contacting the duplex DNA molecule with the MutS homolog, the duplex DNA molecule may be contacted with a membrane, such as a nitrocellulose membrane, under conditions such that double-stranded DNA does not bind to the membrane, but homolog-bound double-stranded DNA does bind to the membrane. Such conditions vary with the type of membrane used and are known in the art. Further by way of example, specific binding of the homolog to the duplex DNA molecule may be detected by comparing the amount of the homolog bound to the duplex DNA molecule with the amount of the homolog bound to other duplex DNA molecules of similar size which are either known to contain mismatches or are known not to contain mismatches. Binding of a greater amount of the homolog to the duplex DNA molecule than the amount that binds to other duplex DNA molecules known not to contain mismatches is an indication that the duplex DNA molecule contains at least one mismatched region. Binding of a lesser amount of the homolog to the duplex DNA molecule than the amount that binds to other duplex DNA molecules known to contain mismatches is an indication that the duplex DNA molecule does not contain a mismatched region.

In a preferred method of detecting specific binding of the MutS homolog to the duplex DNA molecule, an optical affinity biosensor system (OABS) is used to detect specific binding. In an OABS system such as the IAsys™ system (Affinity Sensors, Cambridge, United Kingdom), binding and dissociation events can be detected as one molecule in solution binds to or dissociates from another molecule immobilized on a detector surface of the system. Thus, an OABS may be used to detect specific binding between the MutS homolog and the duplex DNA molecule having a mismatched region in any of the methods of the invention by immobilizing either the homolog or the DNA molecule on the detector surface of the OABS. Specific binding may be differentiated from non-specific binding by comparing

binding of a MutS homolog to a duplex DNA molecule known to comprise a mismatched region and binding of the homolog to a duplex DNA molecule known not to comprise a mismatched region.

The sample nucleotide sequence and the reference nucleotide sequence in the just-described method may be any nucleotide sequence. In one embodiment of the method, the sample nucleotide sequences comprises the sequence of a region of a gene obtained from a first organism and the reference nucleotide sequence comprises the sequence of the region obtained from a second organism. By way of example, the first and the second organism may be members of the same species, such as *Homo sapiens*. In another embodiment of the method, the sample nucleotide sequence comprises the sequence of a region of a gene obtained from an organism and the reference nucleotide sequence comprises a consensus nucleotide sequence of the region. By way of example, the gene may be the human *msh2* gene.

The invention also includes a kit for separating a mismatched duplex DNA molecule from a population of molecules. The kit comprises a MutS homolog, a linker for binding the MutS homolog to a solid support, and an ADP molecule. The kit is useful, for example, for performing the method of the invention of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules, as described herein. To use the kit according to this method, the MutS homolog is contacted with the population of duplex DNA molecules including at least one having a mismatched region, whereby the homolog binds to the mismatched region. The linker is used to bind the homolog to a solid support, either before or after contacting the homolog and the population. After the duplex DNA molecule having a mismatched region is bound to the homolog and the homolog is linked to the solid support, the duplex DNA molecule having a mismatched region is segregated from the population by virtue of being fixed, via the homolog, to the solid support. The solid support may be physically separated from the population if separation of the duplex DNA molecule having a mismatched region and the other DNA molecules of the

population is desired. The kit may further comprise the solid support, such as polystyrene beads or a polystyrene microwell plate.

The methods of the invention include a method of detecting a predisposition of a mammal to carcinogenesis. This method is essentially the same as the method of the invention of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence, except that the sample nucleotide sequence is the nucleotide sequence of a region of a mammalian gene and the reference nucleotide sequence is the consensus nucleotide sequence of the region. The mammalian gene is selected from the group consisting of the *msh2* gene, the *msh3* gene, the *msh6* gene, the *mlh1* gene, the *pms2* gene, the *brca1* gene, the *brca2* gene, the *pten* gene, and the *p53* gene. Detection of a difference between the sample nucleotide sequence and the reference nucleotide sequence is an indication that the mammal is predisposed to carcinogenesis. In a particularly contemplated embodiment, the mammalian gene is the human *msh2* gene the predisposition to carcinogenesis detected by the method is predisposition of a human for hereditary non-polyposis colorectal cancer.

The invention also includes a method of fractionating a population of DNA molecules. This method takes advantage of the ability of a MutS homolog to bind a duplex DNA molecule having a mismatched region in the presence of ADP, and the ability of the homolog to dissociate from the mismatched DNA molecule in the presence of ATP. The method of the invention of fractionating a population of DNA molecules comprises contacting the population with a MutS homolog, segregating the homolog from the population, and thereafter contacting the homolog with a solution comprising an ATP molecule. A mismatched duplex DNA molecule of the population binds to the homolog in the presence of ADP, is separated from the population when the homolog is segregated from the population, and is released from the homolog in the presence of ATP, whereby the population is fractionated. The population may be collected in one or more fractions after segregating the homolog therefrom, and the mismatched duplex DNA molecule may be collected in one or more additional

fractions during or after contacting the homolog with ATP. By way of example, the homolog is fixed to a solid support, the population is contacted with the solid support by contacting the support with a suspension comprising the population, the solid support is rinsed with a solution which does not comprise the population, and then the solid support is rinsed with a solution comprising ATP. In one embodiment, a solution comprising ATP at a concentration that increases over time is provided continuously to the solid support, whereby a first mismatched duplex DNA molecule which is displaced from the homolog at a relatively low concentration of ATP can be collected in a first fraction and a second mismatched duplex DNA molecule which is displaced from the homolog at a relatively high concentration of ATP can be collected in a second fraction. In another embodiment of this method, the solid support is a chromatography medium, such as agarose beads.

The methods of the invention further include a method of selectively amplifying at least one mismatched duplex DNA molecule in a population of duplex DNA molecules. To perform this method, a MutS homolog is contacted with the population in the presence of a solution comprising an ADP molecule, the homolog and a mismatched duplex DNA molecule bound thereto are segregated from the population, and the mismatched duplex DNA molecule is amplified using a PCR technique, whereby each of the at least one mismatched duplex DNA molecule is selectively amplified. The population of duplex DNA molecules may be any duplex DNA molecules, such as a population of duplex DNA molecules made by contacting a nucleic acid derived from an organism with at least one pair of PCR primers which can be used to amplify a region of a gene of the organism and performing PCR using the nucleic acid and the pair of PCR primers. The population may also, for example, be a cDNA library prepared using mRNA obtained from the organism. In one embodiment of this method, the method further comprises adding a first tail sequence to the 3'-end of the coding strand of the mismatched duplex DNA molecule and adding a second tail sequence to the 3'-end of the noncoding strand of the mismatched duplex DNA molecule prior to amplifying the mismatched duplex DNA molecule, and using two

PCR primers, each of which is complementary to either the first or the second tail sequence. The sequences may be added, for example, by incorporating one of the first or the second tail sequences into one primer used to amplify a duplex DNA molecule and the other of the first or the second tail sequences into the other primer used to amplify the duplex DNA molecule. The tail sequences may also be added to the DNA molecule by blunt-end ligation of a hybrid DNA molecule comprising each of the first and the second tail sequences to the duplex DNA molecule, followed by enzymatic cleavage of the tail DNA molecule, whereby a portion of the tail DNA molecule comprising the first tail sequence remains attached to the 3'-end of one strand of the duplex DNA molecule and a portion of the tail DNA molecule comprising the second tail sequence remains attached to the 3'-end of the other strand of the duplex DNA molecule. In another embodiment of this method, the method is repeated at least twice to improve the purity of the mismatched duplex DNA molecules that are amplified.

The invention also include a method of detecting the presence of a genetic polymorphism in a eukaryotic genome. A genetic polymorphism is a difference between the nucleotide sequence of the one copy of a gene in a chromosome and the nucleotide sequence of the other copy of the gene in the same chromosome. This method comprises amplifying a region of each of two copies of the desired gene to yield an amplified first copy and an amplified second copy. Then the first and the second copy are mixed together, denatured and are subsequently incubated under annealing conditions. The annealed mixture is contacted with a MutS homolog in the presence of a solution comprising an ADP molecule, the homolog is separated from the mixture, and the presence or absence of a nucleic acid specifically bound to the homolog is assessed. The presence of a nucleic acid comprising a DNA strand from the first copy and a DNA strand from the second copy and specifically bound to the homolog is an indication of the presence of a genetic polymorphism. Well known PCR technology can be used to amplify each copy of the gene. In one embodiment, the primers used in the PCR procedure for amplifying each copy are complementary to the

intronic region immediately adjacent an exon of the gene. The mixture may be contacted with the MutS homolog using any of the methods described herein.

In the just-described method, detecting the presence or absence of a nucleic acid specifically bound to the MutS homolog can be performed using any known method of detecting the presence of a nucleic acid including, for example, detection of a shift in the ratio of the absorbance of the homolog at 280 nanometers to the absorbance of the homolog at 260 nanometers, whereby an decrease in this ratio is an indication that a nucleic acid is associated with the homolog. Further by way of example, the presence of the nucleic acid can be detected by detecting the presence of a radioactive, fluorescent, or biotinylated label incorporated into the nucleic acid using known methods. A nucleic acid comprising a DNA strand from the first copy of the gene and a DNA strand from the second copy can be detected by sequencing both strands of the nucleic acid and comparing those sequences. If the two strands of the nucleic acid are from the same copy of the gene, then the two strands should be completely complementary. Thus, if the two strands are not completely complementary, then the two strands are derived from different copies of the gene. Identification of one or more regions of non-complementarity between the two strands is an indication of the presence of a genetic polymorphism. Gel mobility shift assays, DNase footprint assays, or OABS detection methods may also be used to detect the presence or absence of the nucleic acid specifically bound to the homolog.

In one embodiment of the method of the invention of detecting the presence of a genetic polymorphism, a nucleic acid specifically bound to the homolog is amplified prior to detecting the presence or absence of a nucleic acid associated with the homolog. This amplification may be accomplished, for example, by repeating the amplification procedure used in the method to amplify the region of the two copies of the desired gene, except that a nucleic acid specifically bound to the homolog is used in place of a nucleic acid derived from the genome of the animal. Amplification of this nucleic acid facilitates easier detection of the presence or absence of a nucleic acid specifically bound to the homolog because the amount of the nucleic acid is increased

relative to the amount of a potentially contaminating nucleic acid not specifically bound to the homolog.

The invention also includes a composition for segregating mismatched duplex DNA molecules from a population of DNA molecules. The composition comprises a MutS homolog bound to a solid support, wherein the support is in liquid contact with a solution comprising an ADP molecule. The composition is used by contacting the population of DNA molecules with the composition and then segregating the composition from the population. Any of the methods described herein for segregating a MutS homolog bound to a solid support from a population of DNA molecules may be used to segregate the composition from the population. By contacting the composition with the population, a mismatched duplex DNA molecule binds to the homolog of the composition. By segregating the composition from the population, the mismatched duplex DNA molecule bound to the composition is segregated from the population. The mismatched duplex DNA molecule may be dissociated from the composition by contacting the composition with a solution comprising an ATP molecule. The solid support for the composition may be any solid support to which a MutS homolog may be bound, such as polystyrene support linked to the homolog by an antibody which is bound to the support and which specifically binds to the homolog, for example. The solid support may have any form including, but not limited to a microwell plate, a porous membrane, a non-porous membrane, an insoluble particle, a chromatography medium, and a gel.

The invention also includes a kit for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence. The kit comprises a pair of primers adjacent to the region for amplifying the region, a duplex DNA molecule having the reference nucleotide sequence, a solid support, a MutS homolog, a linker for binding the homolog to the solid support, and an ADP molecule. The kit is used by amplifying the region using the pair of primers to yield an amplified DNA molecule, mixing the amplified DNA molecule and the duplex DNA molecule having the reference nucleotide sequence to form a mixture. The nucleic acids in the

mixture are denatured and are annealed. The MutS homolog is added to the mixture and is bound to a solid support. The solid support is segregated from the mixture, and the presence or absence of a nucleic acid specifically bound to the homolog is assessed. The presence of a nucleic acid specifically bound to the homolog is an indication that the nucleotide sequence of the region differs from the reference nucleic acid sequence. Well known PCR methods may be used to amplify the region using the primers, and well known molecular biology methods may be used to denature and anneal the nucleic acids in the mixture. The mixture may be formed before or after denaturing the amplified DNA molecule and the duplex DNA molecule having the reference nucleotide sequence. The homolog may be bound to the solid support before or after contacting the mixture with the homolog. Any of the methods described herein for detecting a nucleic acid specifically bound to the homolog may be used with the kit of the invention for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence.

In one embodiment of the kit of the invention for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence, the pair of primers comprises a sense primer having a first tail sequence and an antisense primer having a second tail sequence. In this embodiment, the genomic region is amplified using the pair of primers, and the amplified DNA molecule is mixed, denatured, and annealed with the duplex DNA molecule having the reference nucleotide sequence. The mixture is contacted with the homolog. The homolog is bound to the solid support and is then segregated from the population. Next, the homolog is contacted with a solution comprising ATP or a high salt concentration to cause a mismatched DNA duplex molecule specifically bound to the homolog to be dissociated therefrom. PCR primers complementary to the first tail sequence and the second tail sequence are used to amplify the mismatched DNA duplex molecule. This embodiment has the advantage that amplification of DNA molecules non-specifically bound to the homolog is minimized.

The invention also includes a nonhuman animal which is nullizygous for both the *Msh2* gene and the *p53* gene and methods of making and using the nonhuman animal, as described herein in Example 2.

The invention is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these examples but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example 1. The Human Mismatch Recognition Complex hMSH2/hMSH6 Functions as a Molecular Switch

Adenine nucleotide binding by the human mismatch recognition protein complex formed by association of the hMSH2 and hMSH6 proteins, is a novel molecular switch. The hMSH2/hMSH6 protein complex is "ON" (i.e. it binds to mismatched DNA) in the ADP-bound form, and "OFF" (i.e. it dissociates from and does not bind to mismatched DNA) in the ATP-bound form. The data presented herein establish that the switch is 'turned OFF' by displacement of complex-bound ADP by ATP. ATP-bound complex is recycled to the ADP-bound form, which is capable of binding to mismatched DNA, by intrinsic ATPase activity of the complex.

The materials and methods used in the experiments presented in this Example are now described.

Overexpression and purification of hMSH2-hMSH6

Clones encoding hMSH2 and those encoding hMSH6 have been described (Acharya et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:13629-13634; Fishel et al., 1993, Cell 75:1027-1038). In the experiments described herein, the clone encoding hMSH6 was modified to further encode six histidine residues at the amino terminus of the hMSH6 protein molecule.

hMSH2 and hMSH6 were overexpressed in SF9 insect cells using the pFastBac™ dual expression vector (Gibco BRL, Grand Island, NY) as described in the Bac-to-Bac™ baculovirus expression systems protocol (Gibco BRL, Grand Island,

NY). SF9 cells suspended in approximately 400 milliliters culture medium were infected using the vector, and were then cultured for 48 hours to achieve a cell density of approximately 10^6 SF9 cells per milliliter. The cells contained in 200 milliliter aliquots of SF9 cells were harvested by centrifugation at 200 × g, resuspended in 10 milliliters of buffer A, and frozen at -80°C. Buffer A comprised 300 millimolar NaCl, 20 millimolar imidazole, 25 millimolar HEPES buffer adjusted to pH 7.8 using NaOH, 10% (v/v) glycerol, 0.5 millimolar phenylmethylsulfonylfluoride (PMSF), 0.8 micrograms per milliliter pepstatin, and 0.8 micrograms per milliliter leupeptin.

Cell extracts were prepared by thawing the cells, passing the cells through a 25 gauge needle, and then ultracentrifuging the extract at 40,000 rotations per minute in a Beckman Ti60 rotor for 70 minutes, according to known methods. About 100 milliliters of infected cells yielded approximately 2 milligrams of hMSH2-hMSH6 protein complex. All of the following protein purification procedures in this Example were carried out at 4°C.

The supernatant was applied to a 2 milliliter nickel-NTA Superflow™ column (Qiagen, Chatsworth, CA) at a flow rate of 0.15 milliliters per minute using a Pharmacia FPLC system. The column was washed by passing 35 milliliters of buffer A through the column. After washing the column, the hMSH2/hMSH6 complex was eluted by applying 30 milliliters of buffer A comprising a linear gradient of imidizole to the column and collecting the eluent from the column in fractions, wherein the concentration of imidizole was varied from 20 millimolar to 200 millimolar. The hMSH2/hMSH6 complex eluted in fractions containing approximately 70 millimolar imidizole.

Fractions from the nickel-NTA column which contained peak amounts of the complex were loaded at a flow rate of 0.2 milliliters per minute directly onto a 1 milliliter PBE 94 column (a polybuffer exchange column obtained from Pharmacia, Upsala Sweden) which had been equilibrated with buffer B. Buffer B comprised 300 millimolar NaCl, 25 millimolar HEPES buffer adjusted to pH 7.8 using NaOH, 1 millimolar dithiothreitol (DTT), 0.1 millimolar ethylenediaminetetraacetic acid

(EDTA), 10% (v/v) glycerol, 0.5 millimolar PMSF, 0.8 micrograms per milliliter pepstatin, and 0.8 micrograms per milliliter leupeptin. The PBE 94 column was washed by passing 10 milliliters of buffer B through the column. After washing the column, the hMSH2/hMSH6 complex was eluted by applying 20 milliliters of buffer B comprising a linear gradient of NaCl to the column and collecting the eluent from the column in fractions, wherein the concentration of NaCl was varied from 300 millimolar to 1 molar. The hMSH2/hMSH6 complex eluted from the PBE 94 column in fractions containing approximately 575 millimolar NaCl.

Fractions collected from the PBE 94 column which contained peak amounts of the complex were dialyzed twice for two hours against 2 liters of a solution comprising 100 millimolar NaCl, 25 millimolar HEPES buffer adjusted to pH 7.8 using NaOH, 1 millimolar DTT, 0.1 millimolar EDTA, and 20% (v/v) glycerol. Aliquots of the dialyzed solution containing the complex were frozen using liquid nitrogen and stored at -80°C for several months without detectable loss of activity.

hMSH2, hMSH6, and bovine serum albumin (BSA) contain nearly identical percentages (12%, 14%, and 13%, respectively) of arginine and heterocyclic amino acids, the amino acids known to interact with the Coomassie Brilliant Blue stain. Protein concentration in an aliquot comprising the hMSH2-hMSH6 complex was determined by subjecting a portion of the aliquot to SDS-PAGE using a 6% (w/v) acrylamide gel, subjecting a known amount of BSA (Boehringer Mannheim, Indianapolis, IN) to SDS-PAGE using a 6% (w/v) acrylamide gel, staining the SDS-PAGE gels with Coomassie Brilliant Blue, and comparing the intensities of the protein bands in the gels to a BSA standard on a Coomassie stained 6% SDS PAGE to calculate protein concentration. The intensities of stained protein bands were measured using BioRad Gel Doc and Molecular Analyst™ software. This protein quantitation method revealed the hMSH2 and hMSH6 proteins to be in near exact equimolar proportion in the complex formed between the two proteins.

Preparation of 39- and 81-base pair oligonucleotide probes

The sequence of the 39-base pair oligonucleotide used in the experiments presented in this Example was: 5'-CGG CGA ATT CCA CCA AGC TTG ATC GCT CGA GGT ACC AGG-3' (SEQ ID NO:1). The homologous 39-base pair DNA substrate used in the experiments presented in this Example was made by annealing the 39-base pair oligonucleotide with an oligonucleotide (SEQ ID NO:2) which was completely complementary thereto. The G/T mismatched 39-base pair DNA substrate used in the experiments presented in this Example was made by annealing the 39-base pair oligonucleotide with an oligonucleotide (SEQ ID NO:3) which was completely complementary thereto, except that the oligonucleotide contained a G residue at the nucleotide position complementary to the T residue at position 20 (numbered in the direction extending from the 5' end to the 3' end) of the 39-base pair oligonucleotide. SEQ ID NO: 2 and SEQ ID NO: 3 are listed in Figure 10.

The nucleotide sequence of the 81-base pair oligonucleotide used in the experiments described in this Example was: 5'-AAA GCT GGA GCT GAA GCT TAG CTT AGG ATC ATC GAG GAT CGA GCT CGG TGC AAT TCA GCG GTA CCC AAT TCG CCC TAT AGT-3' (SEQ ID NO: 4). The homologous 81-base pair DNA substrate used in the experiments presented in this Example was made by annealing the 81-base pair oligonucleotide with an oligonucleotide (SEQ ID NO: 5) which was completely complementary thereto. The G/T mismatched 81-base pair DNA substrate used in the experiments presented in this Example was made by annealing the 81-base pair oligonucleotide with an oligonucleotide (having the nucleotide sequence listed in SEQ ID NO: 6) which was completely complementary thereto, except that the oligonucleotide contained a T residue at the nucleotide position complementary to the G residue at position 41 (numbered in the direction extending from the 5' end to the 3' end) of the 81-base pair oligonucleotide. SEQ ID NO: 5 and SEQ ID NO: 6 are listed in Figure 10.

^{32}P-end-labeled DNA substrates were prepared by incubating single stranded oligonucleotides in the presence of T4 polynucleotide kinase (Promega Corp., Madison, WI) and [^{32}P]γ-ATP (NEN Dupont, Wilmington, DE). Excess label was separated from the labeled DNA substrates using a Centrisep™ column (Princeton Separations, Princeton, NJ) per the manufacturer's instructions.

Labeled DNA substrate was annealed with a single-stranded DNA molecule which was either completely complementary thereto or contained a single G/T mismatch. To anneal the labeled DNA substrate with the single-stranded DNA molecule, the labeled molecule was suspended in a solution comprising a 10-fold excess of the single-stranded DNA molecule, 10 millimolar Tris buffer which had been adjusted to pH 7.5 using HCl, 100 millimolar NaCl, and 1 millimolar EDTA. The suspension was heated to 95°C and then slowly cooled to 55°C and was maintained at this temperature for twelve hours. Single-stranded DNA was removed from the suspension by incubating the suspension with benzoylated naphthoylated DEAE cellulose (BND cellulose, Sigma Chemical Co., St. Louis, MO) for twenty minutes in the presence of a solution comprising 1.5 molar NaCl, 20 millimolar Tris buffer which had been adjusted to pH 7.5 using HCl, and 0.5 millimolar EDTA. BND cellulose was then pelleted by centrifuging the suspension for about five minutes using an Eppendorf bench-top centrifuge. Double-stranded DNA, which remained in the supernatant, was separated from the BND cellulose by filtration and was then precipitated by adding ethanol to the supernatant. The double-stranded labeled DNA substrate was resuspended in a solution comprising 10 millimolar Tris buffer which had been adjusted to pH 7.5 using HCl, 100 millimolar NaCl, and 1 millimolar EDTA. Single-stranded DNA could not be detected in the solution, as assessed by 4% (w/v) native PAGE separation of the nucleotides in the solution. Non-^{32}P-labeled oligonucleotides were prepared using analogous methods.

Gel mobility shift assays

Gel mobility shift assays were performed by incubating a complex comprising an hMSH2 protein molecule and an hMSH6 protein molecule and 9

femtomoles of either the ^{32}P-labeled homologous 81-base pair DNA substrate or the ^{32}P-labeled G/T-mismatched 81-base pair DNA substrate in a buffer comprising 50 millimolar NaCl, 25 millimolar HEPES buffer which had been adjusted to pH 7.5 using NaOH, 1 millimolar DTT, 0.01 millimolar EDTA, and 15% (v/v) glycerol. The buffer included 10 nanograms per microliter of poly dI-dC (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Poly dI-dC is an alternating nucleic acid polymer which does not interfere with binding of the hMSH2/hMSH6 complex to DNA. In certain experiments described herein, the incubation mixture further comprised selected concentrations of nucleotides or non-labeled DNA. In other experiments described herein, the incubation mixture further comprised 1 millimolar $MgCl_2$ or 5 millimolar EDTA. Except as otherwise described herein, each incubation mixture had a volume of 20 microliters and was incubated for fifteen minutes at 37°C and then immediately placed on ice. Each incubation mixture was applied to a gel comprising 4% (w/v) polyacrylamide (29:1 ratio of acrylamide:*bis*-acrylamide) 4% (v/v) glycerol, 40 millimolar Tris acetate buffer (pH 7.8), and 1 millimolar EDTA. Electrophoresis was performed by applying 200 volts to the gel for two hours. Following electrophoresis, each gel was dried and quantitated using a phosphoimaging device obtained from Molecular Dynamics.

Footprint assays

Incubation of the hMSH2/hMSH6 complex with ^{32}P-labeled DNA substrates was performed as described for gel mobility shift assays, except that 18 femtomoles of ^{32}P-labeled DNA substrate was used in each assay. Following incubation, 80 microliters of a buffer comprising 50 millimolar NaCl, 25 millimolar HEPES buffer which had been adjusted to pH 7.8 using NaOH, 1 millimolar DTT, 10 nanograms per microliter poly dI-dC, 1.25 millimolar $CaCl_2$, 3.1 millimolar $MgCl_2$, 10% (v/v) glycerol, and 33 picograms per microliter DNase (Boehringer Mannheim, Indianapolis, IN) was added to each incubation mixture. The mixtures were incubated at 37°C for an additional three minutes, and then 0.7 milliliters of a solution having a pH of 5.2 and comprising 95% (v/v) ethanol and 180 millimolar sodium acetate was

added to each mixture to halt the DNase reaction and to precipitate the nucleic acids present in the mixture.

DNase-treated nucleic acids were resuspended in 4 microliters of a solution comprising 80% (v/v) formamide, 10 millimolar NaOH, 1 millimolar EDTA, and 0.1 % (w/v) bromophenol blue. The suspension was heated at 90°C for five minutes and was applied to a gel comprising 8% (w/v) polyacrylamide (29:1 ratio of acrylamide:*bis*-acrylamide), 90 millimolar tris-borate buffer (pH 8), and 2 millimolar EDTA. Following electrophoresis for 2 hours at 200 volts, each gel was dried and imaged on a phosphoimaging device. Individual bases of the 81-base pair DNA substrates were identified by Maxam-Gilbert sequencing reactions performed as described (Ausubel et al., 1994, Current Protocols in Molecular Biology, 8th Ed., Janssen, ed., John Wiley & Sons, Inc., Boston).

ATPase assays

ATPase activity was measured in a reaction mixture comprising 20 microliters of Buffer P, 500 micromolar non-labeled ATP (except where indicated), and 16.5 nanomolar [^{32}P]γ-ATP. Buffer P comprised 40 millimolar HEPES which had been adjusted to pH 7.8 using NaOH, 75 millimolar NaCl, 10 millimolar $MgCl_2$, 1.75 millimolar DTT, and 0.075 millimolar EDTA, and 15 % (v/v) glycerol. Steady state reaction measurements were made using 60 nanomolar hMSH2/hMSH6 complex and either 240 nanomolar homoduplex 39-base pair DNA substrate or 240 nanomolar G/T mismatched 39-base pair DNA substrate. Reaction mixtures were incubated at 37°C for thirty minutes, and the reaction was stopped by addition of 400 microliters of a solution comprising 10% (w/v) activated charcoal (Sigma Chemical Co., St. Louis, MO) and 1 millimolar EDTA. Charcoal was pelleted by centrifuging the mixture at 10,000 rotations per minute for ten minutes. The ^{32}P content of duplicate 100 microliter aliquots of the supernatant was assessed by liquid scintillation.

Initial velocity measurements were made by incubating the hMSH2/hMSH6 complex for ten minutes at 25°C in a reaction mixture comprising one volume Buffer P containing no $MgCl_2$, 200 nanomolar non-labeled ATP, and 16.5

nanomolar [^{32}P]γ-ATP. To start the reaction, an equal volume of buffer P comprising 20 millimolar $MgCl_2$ and 1 millimolar non-labeled ATP was mixed with the reaction mixture, which raised the $MgCl_2$ and ATP concentrations to 10 millimolar and 500 micromolar, respectively. Aliquots were removed at selected times and electrophoresed as described herein. A control aliquot was removed and prepared for electrophoresis prior to addition of the $MgCl_2$-containing Buffer P to the reaction mixture.

ADP exchange assays

The ADP-ATP exchange rate was determined in a reaction mixture which comprised Buffer Q, 2.3 micromolar [^{3}H]-ADP, and 60 nanomolar hMSH2/-hMSH6 complex. Buffer Q comprised 25 millimolar HEPES which had been adjusted to pH 7.8 using NaOH, 75 millimolar NaCl, 10 millimolar $MgCl_2$, 1 millimolar DTT, and 15% (v/v) glycerol. This reaction mixture was incubated for ten minutes at room temperature. 240 nanomolar G/T-mismatched 39-base pair DNA substrate was added to the reaction mixture, and the incubation was continued for an additional ten minutes. The final volume of the reaction mixture was 10 microliters. The order of addition of DNA and ADP did not affect the kinetic results obtained using this assay. An equal volume Buffer Q comprising 1 millimolar non-labeled ATP was then added to the reaction mixture. Reactions were incubated at 25°C for a selected time and then halted by diluting the reaction mixture with 4 milliliters of an ice-cold stop buffer comprising 25 millimolar HEPES which had been adjusted to pH 7.8 using NaOH, 100 millimolar NaCl, and 10 millimolar $MgCl_2$.

Each halted reaction mixture was immediately filtered on a HAWP nitrocellulose membrane (Millipore, Bedford, MA) and washed thrice with 4 milliliters of the ice-cold stop buffer. Each filter was air dried and incubated overnight in a standard scintillation cocktail. Radioactivity retained on the filters was quantified using a Beckman scintillation counter. A control reaction mixture was prepared by not adding the Buffer Q comprising 1 millimolar non-labeled ATP to the reaction mixture. The amount of [^{3}H]-ADP retained on the membrane to which the control reaction

mixture was applied was considered to correspond to the amount of radioactivity retained when 100% of the complex had [^{3}H]-ADP bound thereto.

Thin Layer Chromatography (TLC) Analysis

TLC was used to determine the composition of an ATPase reaction mixture which was prepared as described herein in the presence of the G/T-mismatched 39-base pair DNA substrate, 15 micromolar ATP, and 0.01 micromolar [^{32}P]α-ATP and which was permitted to react for twenty minutes at 37°C. TLC was performed as previously described (Fishel et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:36-40).

The results of the experiments presented in this Example are now described.

Overexpression and purification of the hMSH2-hMSH6 protein complex

hMSH2 and hMSH6 proteins were overexpressed in insect cells using a dual expression baculovirus vector, as indicated by the proteins found cell extract (Figure 1). Co-expression of hMSH2 and hMSH6 proteins resulted in formation of a completely soluble protein complex. Independent expression of either protein alone resulted in formation of a substantial amount of insoluble protein product. hMSH2 and hMSH6 likely exist together as a highly stable complex *in vivo*, as judged by the results obtained in the Experiments described in this Example, the ability of investigators to copurify these two proteins from human cells (Drummond et al., 1995, Science 268:1909-1912), and the ability of these two proteins to interact *in vitro* (Acharya et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:13629-13634).

Purification of hMSH2 and hMSH6 from insect cells indicated that a stable heterodimer of the two proteins had been formed. Quantitative densitometry of Coomassie-stained products consistently revealed that the hMSH2 and hMSH6 subunits were present in an equimolar ratio, as was observed with the yeast MSH2/MSH6 protein complex (Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447). The purification methodology described herein yielded a protein preparation which was more than 95% homogeneous, which exhibited high MSH2/MSH6 activity, and which appeared to be free of any contaminating nucleic acid or nucleotide.

G/T mismatch binding by hMSH2-hMSH6 is a model for mismatch recognition

The hMSH2-hMSH6 protein complex has been demonstrated herein and by others to bind to the eight possible mismatched nucleotide combinations, as well as to a subset of single nucleotide insertion/deletion mismatches (Acharya et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:13629-13634; Drummond et al., 1995, Science 268:1909-1912; Hughes et al., 1992, J. Biol. Chem. 267:23876-23882). The G/T mismatch was chosen as a model for quantitative analysis of hMSH2-hMSH6 mismatch binding because of its apparently intermediate-to-high recognition specificity, as indicated, for example, by the data presented in Figures 2A-2D.

The apparent dissociation constant (K_d) was determined in a simple buffer system comprising neither an adenine nucleotide nor magnesium using the homologous 81-base pair DNA substrate and the G/T-mismatched 81-base pair DNA substrate described herein. Results obtained using both gel shift assays, as depicted in Figure 2A, and DNase footprint assays, as depicted in Figure 2C, indicated that K_d of the hMSH2/hMSH6 complex for G/T mismatches was 20 ± 5 nanomolar. Binding of non-mismatched DNA to the complex was not saturable, even at homoduplex concentrations greater than 400 nanomolar.

The binding of the hMSH2/hMSH6 complex to a G/T mismatch is at least ten times more efficient than binding of hMSH2 alone to the G/T mismatch (Fishel et al., 1994, Science 266:1403-1405; Fishel et al., 1994, Cancer Res. 54:5539-5542; Mello et al., 1996, Chemistry & Biology 3:579-589). This observation indicates that formation of the protein complex enhances both the affinity and the specificity of hMSH2-binding to mismatched DNA (Acharya et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:13629-13634).

Gel mobility shift assays performed using the G/T-mismatched 39-base pair DNA substrate described herein or using the G/T-mismatched 81-base pair DNA substrate and a buffer comprising 2 millimolar $MgCl_2$ yielded results similar to those

shown in Figure 2A. The hMSH2-hMSH6 complex appears to bind G/T mismatched DNA in multiple forms which are differentiable by gel mobility shift assay.

DNase footprint analysis of hMSH2/hMSH6 complex binding to the G/T-mismatched 81-base pair DNA substrate indicated that the complex asymmetrically protects about 25 nucleotides on both strands of the substrate. As shown in Figure 2C, there appeared to be two domains protected by the complex from cleavage by DNase. One domain appeared to be centered on the G/T mismatch in the substrate. The other domain was adjacent the domain centered on the G/T mismatch and was separated from that domain by a single DNase-sensitive nucleotide. These data are qualitatively similar to those observed in similar experiments using the *E. coli* and *T. aquaticus* MutS proteins (Su et al., 1986, Proc. Natl. Acad. Sci., U.S.A. 83:5057-5061; Su et al., 1988, J. Biol. Chem. 263:6829-6835; Biswas et al., 1997, J. Biol. Chem. 272: 13355-13364).

Although a shifted complex could be detected by gel mobility shift assay using homoduplex DNA, no specific DNase footprint could be identified, as indicated by the data presented in Figure 2D. Lack of saturatability and lack of a specific footprint are consistent with the ability of the hMSH2/hMSH6 complex to weakly and non-specifically associate with homoduplex DNA.

Shifted complexes formed between the complex and homoduplex DNA and those formed between the complex and G/T-mismatched DNA migrated differently in gel mobility shift assays, as shown in Figures 2A and 2B. Homoduplex DNA-bound complex (designated 'NS' for 'non-specific' in Figure 2B) migrated more slowly than G/T-mismatched DNA-bound complex (designated 'S' for 'specific' in Figure 2A). These results suggest that homoduplex DNA-bound complex adopts a different conformation than mismatched DNA-bound complex. Alternatively, there may have been a greater quantity of the complex bound to homoduplex DNA than to mismatched DNA.

When the homoduplex 39-base pair DNA substrate described herein was contacted with the complex, no NS product was observed in the gel mobility shift

assay. The DNA length dependence of NS product formation may result if a minimum number of base pairs were necessary to assume an alternative DNA and/or hMSH2- or hMSH6-protein conformation or to bind multiple hMSH2/hMSH6 molecules.

These results demonstrate the high specificity of complex binding to the G/T-mismatched 81-base pair DNA substrate. The binding was found to be quantitatively similar by both gel mobility shift and footprint analysis. In addition, a low level non-specific binding to duplex DNA was observed and found to be easily distinguished via its altered mobility using gel mobility shift analysis.

The hMSH2-hMSH6 complex converts ATP to ADP in the presence of mismatched DNA

Both bacterial and yeast MutS homologs have been shown to possess intrinsic low-level ATPase activity (Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447; Chi et al., 1994, J. Biol. Chem. 269: 29993-29997; Chi et al., 1994, J. Biol. Chem. 269:29984-29992; Habe et al., 1988, J. Bacteriol. 170:197-202). There are conflicting reports regarding the capacity of mismatched heteroduplex and/or homoduplex DNA to stimulate this intrinsic ATPase activity (Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447; Chi et al., 1994, J. Biol. Chem. 269: 29993-29997; Chi et al., 1994, J. Biol. Chem. 269:29984-29992).

It has been demonstrated herein that the hMSH2/hMSH6 complex possesses intrinsic DNA-dependent ATPase activity that is dependent upon the presence of magnesium as a cofactor, as indicated by the results illustrated in Figure 3A. Saturation of the ATPase activity by hMSH2-hMSH6 at protein concentrations above 0.6 micromolar are the likely result of limiting DNA, which was introduced at a fixed concentration of 240 nanomolar.

As indicated by the results presented in Figure 3B, thin layer chromatography revealed that the hMSH2-hMSH6 ATPase uniformly converts ATP to ADP and inorganic phosphate. Using Lineweaver-Burk analysis and Eadie-Hofstee analysis, it was determined that hMSH2/hMSH6 complex ATPase is most active in the presence of a G/T mismatch (Figure 3C). The value of k_{cat} using ATP and G/T-

mismatched DNA as substrates was about 26 minute^{-1}. The value of K_m using ATP and G/T-mismatched DNA as substrates was about 46 micromolar. hMSH2/hMSH6 complex ATPase is substantially less active in the presence of homoduplex DNA. The value of k_{cat} using ATP and G/C-mismatched DNA as substrates was about 7.4 minute^{-1}. The value of K_m using ATP and G/C-mismatched DNA as substrates was about 23 micromolar. hMSH2/hMSH6 complex ATPase is substantially inactive in the absence of DNA. The value of k_{cat} using ATP alone as a substrate was about 0.9 minute^{-1}. The value of K_m using ATP alone as a substrate was about 10 micromolar.

ATPase activity stimulation was the same regardless of whether the homoduplex DNA had a length of 39 base pairs, 81 base pairs or 2,900 base pairs, and was also the same regardless of whether the mismatched DNA had a length of 39 base pairs or 81 base pairs. These results indicated that hMSH2/hMSH6 complex ATPase activity is not dependent upon DNA length.

It was observed that k_{cat} using ATP alone as a substrate was lower than k_{cat} using ATP and homoduplex DNA as a substrate and this value was lower than k_{cat} using ATP and mismatched DNA as substrates. However, K_m for ATP in the absence of DNA was lower than K_m for ATP in the presence of homoduplex DNA, and this value was lower than K_m for ATP in the presence of mismatched DNA. These observations indicated that although the rate of hydrolysis is increased in the presence of a mismatch, the affinity for ATP is decreased. These results are qualitatively similar to the phenomenon of uncompetitive inhibition which may be ascribed to the presence of independent and separate binding sites as well as a ping-pong binding mechanism (Dixon et al., 1979, Enzymes, 3rd Ed., Academic Press, New York). Single-stranded DNA (ssDNA) was found to be the most potent stimulator of hMSH2/hMSH6 ATPase activity. Thus, the conflicting reports in the prior art regarding ATPase activities of related MutS homologues may have resulted from contamination by ssDNA leached from columns used to purify the homologues and/or non-annealed ssDNA that remained following preparation of oligonucleotide substrates.

hMSH2/hMSH6 mismatch binding is abolished in the presence of ATP in the absence of hydrolysis of ATP

Both bacterial and eukaryotic MutS homologs fail to form a specific complex with a mismatched oligonucleotide in the presence of ATP (Drummond et al., 1995, Science 268:1909-1912; Haber et al., 1991, EMBO. J. 10:2707-2715; Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447; Grilley et al., 1989, J. Biol. Chem. 264:1000-1004). Before the present invention, it was believed that ATP hydrolysis catalyzed by MutS protein drove translocation of the protein along a duplex DNA strand, causing dissociation of the protein from any mismatch with which it might be associated (Grilley et al., 1989, J. Biol. Chem. 264:1000-1004; Modrich, 1989, J. Biol. Chem. 264:6597-6600; Modrich, 1991, Annu. Rev. Genet. 25:229-253; Modrich et al., 1996, Annu. Rev. Biochem. 65:101-133; Allen et al., 1997, EMBO Journal 16:4467-4476). The suggestion that ATP hydrolysis was required for the mismatch release was based on the observation by others that adenylyl-imidodiphosphate (AMP-PNP), a non-hydrolyzable analog of ATP, does not alter mismatch binding (Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447; Drummond et al., 1995, Science 268:1909-1912).

The experiments described in this Example establish that the hMSH2/hMSH6 complex is released from a G/T-mismatched DNA substrate in the presence of ATP (Figures 4A and 4D. The value of IC_{50} (the concentration of ATP required to cause release of 50% of a population of complexes from a G/T-mismatched DNA substrate) was determined to be approximately 3 micromolar. Adenosine-5'-O-3-thiotriphosphate (ATP-γ-S), a poorly-hydrolyzable ATP analog (Sekimizu et al., 1987, Cell 50:259-265; Yu et al., 1992, J. Mol. Biol. 225:193-216), caused a similar release of the hMSH2/hMSH6 complex from a G/T-mismatched DNA substrate, the value of IC_{50} for ATP-γ-S being 3 micromolar (Figures 4B and 4D). Addition of ADP to the mismatch binding reaction mixture resulted increased binding affinity of the complex for the G/T-mismatched DNA substrate (Figures 4C and 4D.

The results presented in this Example demonstrate that release of the hMSH2/hMSH6 complex from a G/T-mismatched DNA substrate to which it is bound

is not associated with ATP hydrolysis. This conclusion follows from the observations that release of the complex occurs in the absence of exogenous magnesium and that release of the complex from the substrate is effected by the presence of ATP-γ-S regardless of the presence or absence of magnesium. The presence of magnesium is absolutely required for hMSH2-hMSH6 dependent ATP hydrolysis. Furthermore, NS binding of hMSH2 to homoduplex DNA is insensitive to the addition of exogenous ATP. Thus, the presence of ATP affects only the ability of the hMSH2/hMSH6 protein complex to bind to mismatched DNA substrates. Binding of the complex to homoduplex DNA is not affected by ATP.

The presence of 2'-deoxy adenosine triphosphate (dATP) to the mismatch binding reaction mixture caused release of a G/T-mismatched DNA substrate from the hMSH2/hMSH6 protein complex, similarly to the release caused by the presence of ATP or ATP-γ-S in the mixture, as illustrated in Figure 5. No other nucleotide was found to stimulate the release of the G/T-mismatched DNA substrate from the complex.

Neither of two other non-hydrolyzable analogs of ATP, namely AMP-PNP and adenyl(β, γ-methylene)diphosphonate (AMP-PCP), caused release of the complex from the substrate. Equilibrium competition between each of these two analogs and ATP suggested that they bind to the complex and caused effects similar to those caused by ADP. Failure of AMP-PNP and AMP-PCP to stimulate release of mismatched DNA from the complex demonstrated that the interaction between the β-γ bridging oxygen atom of ATP and either the complex or the mismatched DNA substrate bound to the complex are for release of the substrate from the complex. Enzyme-nucleotide triphosphate complexes in which the β, γ oxygen atom interacts with either the enzyme or its substrate are not unknown. For example, the Ras GTPase binds GTP, and donation of a hydrogen bond to the β-γ bridging oxygen of GTP is thought to contribute to catalysis by the enzyme (Maegley et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:8160-8166).

The results presented in this example demonstrate that the hMSH2/hMSH6 complex binds to a mismatched DNA substrate in the presence of ADP, and that the substrate is released from the complex in the presence of ATP or dATP. Because ATP-induced release of the substrate from the complex does not require magnesium and is similarly induced by ATP-γ-S, ATP hydrolysis is not implicated in substrate release. As increasing amounts of ATP or ATP-γ-S were added to the mismatch binding reaction mixture, approximately 15% of S-shifted material gradually became reassociated with the DNA in the form of a NS-shifted complex (Figures 4A and 4B). This fraction was consistent with the amount of NS binding observed for homoduplex DNA at this concentration of the complex (Figure 2B). These results indicated that hMSH2/hMSH6 complex molecules which dissociated from mismatched substrate could reassociate with either the duplex arms or the ends of the substrate.

ATP hydrolysis catalyzed by the hMSH2/hMSH6 complex results in recovery of mismatch binding activity of the complex

To determine the role of ATP hydrolysis in mismatch recognition, ATP or ATP-γ-S was introduced into a mismatch binding reaction mixture in the absence of magnesium. As illustrated in Figures 4A, 4B, 4D, and 5, introduction of either compound resulted in release of the hMSH2/hMSH6 complex from the mismatched DNA substrate in the absence of hydrolysis of the compound. In experiments presented in Figures 6A, 6B, and 6C, magnesium was added to each reaction mixture, which was maintained at 37°C, and the G/T mismatch binding activity of hMSH2-hMSH6 was followed over time, with time zero corresponding to the time at which magnesium was added. In the reaction mixture comprising ATP, mismatched DNA substrate binding activity of the complex was initially low, nearly 70% of this activity was recovered after ten minutes of incubation at 37°C, and more than 95% of the activity was recovered fifty minutes after magnesium addition. Substantially less (about 22%) of mismatched DNA substrate binding activity was recovered in the reaction mixture to which ATP-γ-S was added. These results demonstrated that

efficient hydrolysis by the complex is essential for recovery of the complex's mismatch binding activity. Substitution of ATP with dATP produced quantitatively similar recovery of mismatch binding activity (i.e. >95% recovery) following incubation at 37°C. Taken together, these results demonstrated that the intrinsic ATPase activity associated with the human hMSH2/hMSH6 complex is required for recovery from mismatch-release induced by binding to and/or exchange with, ATP or dATP.

Complete recovery of mismatched DNA substrate binding activity of the hMSH2/hMSH6 complex, which activity was abolished by exposing the complex to ATP, was achieved by increasing the ratio of the concentration of ADP to the ratio of ATP in the solution in which the complex was suspended (Figures 6D and 6E). In this competition experiment, mismatch binding reaction mixtures comprised 0.2 millimolar ATP, 1 millimolar $MgCl_2$, and a selected concentration of ADP from 0 to 3.2 millimolar. It was determined that a 2- to 3-fold excess of ADP to ATP resulted in reversal of approximately half of the release of substrate by the complex caused by the presence of ATP. Approximately complete reversal of substrate release caused by the presence of ATP was achieved by providing a 16-fold excess of ADP to the mixture. A qualitatively similar, though functionally opposite, result was obtained when the competition was performed by including a fixed concentration of ADP in the reaction mixture and adding various concentrations of ATP. Thus, ADP and ATP are nearly equivalent in their ability to associate with the hMSH2/hMSH6 complex, but the two nucleotides elicited opposite functional effects on mismatch binding. ATP caused release of substrate bound to the complex, and ADP induced binding of the substrate to the complex. Therefore, ADP is responsible for mismatch binding recovery.

Taken together, these observations support the conclusion that the hMSH2/hMSH6 complex functions as a molecular switch, wherein the ATP- (or dATP-) bound complex is "OFF" (i.e. unable to bind a mismatched DNA substrate) and the ADP-bound complex is "ON" (i.e. able to bind a mismatched DNA substrate). A model of the role of the hMSH2/hMSH6 complex is illustrated in Figure 9.

ATP hydrolysis and ADP-ATP exchange determine mismatch binding functions of the hMSH2/hMSH6 complex

Steady-state analysis of an enzyme having ATPase activity reflects the rate-limiting step of the reaction, which can be either γ-phosphate hydrolysis or adenine nucleotide exchange, as indicated in Figure 7A. To understand the mechanism of the ATPase activity exhibited by the hMSH2/hMSH6 protein complex and to further define the rate-limiting steps, both hydrolysis and nucleotide exchange steps were directly examined.

Initial rate (i.e. single-turnover) analysis of an enzyme which exhibits ATPase activity involves direct examination of the rate of γ-phosphate hydrolysis, and was performed using a method which is similar to that used for the examination of regulators of G-protein signaling (RGS; Dohlman et al., 1997, J. Biol. Chem. 272:3871-3874). In these experiments, 0.2 micromolar [^{32}P]γ-ATP was contacted with hMSH2/hMSH6 protein complex in the absence of magnesium, yielding a complex having a [^{32}P]γ-ATP molecule bound thereto. At a selected time, magnesium and an excess of non-labeled ATP were added to the reaction mixture, and the rate of a single-round of γ-phosphate hydrolysis was assessed. Subsequent rounds of hydrolysis were undetectable because the ATP hydrolyzed during those rounds was not labeled. Because the calculated K_{cat} for ATP at 37°C was in excess of 20 minute^{-1}, and because this rate was above the limit of detection of this methodology, these initial rate experiments were performed at 20°C. It was determined that the hMSH2/hMSH6 complex rapidly hydrolyzed ATP in either the presence or the absence of DNA. These results indicated that γ-phosphate hydrolysis was not the rate limiting step in the steady-state ATP hydrolysis by the complex.

The extent of ATP hydrolysis which could be detected was equivalent to the total number of hMSH2/hMSH6 complex molecules which could be bound to ^{32}P-labeled ATP prior to the addition of magnesium. The maximal extent of detectable ATP hydrolysis was determined to depend on the amount of the G/T-mismatched DNA substrate present in the reaction mixture during binding of labeled ATP to the complex

(Figures 7B and 7C). When the concentration of the G/T-mismatched DNA substrate in to the reaction mixture exceeded the apparent K_d for G/T-mismatched DNA substrate (i.e. about 20 nanomolar), the maximal extent of ATP hydrolysis decreased (Figure 7C). This observation indicated that binding of the hMSH2/hMSH6 protein complex to a mismatched DNA molecule prior to binding of ATP to the complex inhibits binding of ATP to the mismatched DNA-bound complex. This observation is consistent with the pseudo-uncompetitive behavior deduced in the steady-state ATPase activity experiments described herein (Dixon et al., 1979, Enzymes, 3rd Ed., Academic Press, New York).

Adenine nucleotide exchange was assessed using a method similar to that used for guanine nucleotide exchange experiments involving G proteins. In these studies, [^{3}H]-ADP was contacted with hMSH2/hMSH6 protein complex in the presence of magnesium, yielding [^{3}H]-ADP-bound complex. At a selected time, an excess of non-labeled ATP was added to the reaction mixture, and the amount of ADP that remained bound to the complex was assessed at selected times.

In the absence of DNA, incomplete ADP nucleotide exchange was observed during a 15 minute reaction period. The half-life of the ADP-bound complex was greater than eight hundred seconds (Figure 7D. These results clearly suggest that in the absence of DNA, replacement of ADP by ATP is the rate limiting step for the hMSH2/hMSH6 complex ATPase activity.

In the presence of G/T-mismatched DNA substrate, nucleotide exchange was significantly more rapid, the half-life of the ADP-bound complex being less than two seconds, as indicated by the data depicted in Figure 7D. Thus, it was demonstrated that binding of the complex to a G/T-mismatched DNA substrate stimulated replacement of the labeled ADP molecule originally bound to the complex by a non-labeled ATP molecule.

Taken together with the results obtained from the single turnover hydrolysis experiments described herein, these observations indicated that in the absence of mismatched DNA, the hMSH2/hMSH6 protein complex is capable of a

single ATP hydrolysis reaction that yields an ADP-bound complex. While in the ADP-bound form, the complex does not exchange ADP for ATP until the complex binds to a DNA mismatch. By binding to a mismatch, the ADP-bound complex becomes competent to exchange ADP for ATP. Exchange of ADP for ATP causes release of the complex from the mismatch. ATP-bound complex, no longer bound to mismatched DNA, is capable of catalyzing ATP hydrolysis, yielding ADP-bound complex, which is competent to bind to a DNA mismatch. These results indicate that the hMSH2/hMSH6 protein complex is a molecular switch controlled by the phosphorylation state of the adenine nucleotide bound thereto.

Release of the hMSH2/hMSH6 protein complex from a G/T-mismatched DNA substrate occurs by simple dissociation

Prior art models of mismatch recognition by MutS homologs implicated ATP-dependent translocation and/or treadmilling along DNA as a mechanism for association and dissociation of the homolog with a DNA mismatch (Modrich, 1989, J. Biol. Chem. 264:6597-6600; Modrich, 1991, Annu. Rev. Genet. 25:229-253; Modrich et al., 1996, Annu. Rev. Biochem. 65:101-133; Allen et al., 1997, EMBO Journal 16:4467-4476). Common to all of these prior art models is a postulated time-dependent unidimensional homolog displacement mechanism which occurs whether the homolog is bound to duplex DNA or mismatched DNA. In contrast, a simple dissociation mechanism would exhibit rapid and two-dimensional displacement of the homolog from duplex DNA or mismatched DNA.

The ability to distinguish NS and S electrophoretic bands corresponding to the homologous 81-base pair DNA substrate-bound complex and the G/T-mismatched 81-base pair DNA substrate-bound complex (Figure 4A) provided an opportunity to examine the dissociation mechanism of the hMSH2/hMSH6 protein complex from the G/T-mismatched DNA substrate, as well as from homoduplex DNA. In these experiments, the G/T-mismatched DNA substrate was bound to hMSH2/hMSH6 substrate, and an excess of an unlabeled competitor DNA or an excess of ATP, or both, was added to the mixture. If a tracking or sliding mechanism of the

prior art were operable for hMSH2/hMSH6 complex dissociation, it would be expected that a time-dependent loss of the S shifted electrophoretic band of G/T-mismatched DNA substrate-bound complex would be observed, and that a coincident gain of the NS electrophoretic band would be observed. If a simple dissociation mechanism were operable for hMSH2/hMSH6 complex dissociation, it would be expected that loss of the S shifted band would be observed without any coincident increase in the intensity of the NS shifted band because the vast excess of unlabeled homoduplex DNA would preclude secondary reassociation of the complex with the arms or ends of the labeled G/T-mismatched DNA substrate.

Three experiments were performed to determine the mechanism of hMSH2/hMSH6 protein complex dissociation from a labeled 81-base pair G/T-mismatched DNA substrate. The results of these experiments are illustrated in Figure 8.

In the first experiment, the stability of G/T-mismatched DNA substrate-bound hMSH2/hMSH6 complex was assessed by exposing the mismatched substrate-bound complex to a 400-fold excess of non-labeled homoduplex DNA and observing the intensities of S shifted and NS shifted electrophoretic bands at selected times (Figure 8C). Examination of the gel depicted in Figure 8C indicated that the S-shifted electrophoretic band, and thus the amount of the G/T-mismatched DNA substrate-bound hMSH2/hMSH6 complex in the reaction mixture, was not reduced significantly over the ten minute incubation period. Thus, the half-life of the G/T-mismatched DNA substrate-bound hMSH2/hMSH6 complex was much greater than ten minutes, meaning that the mismatched substrate-bound complex is stable in the presence of a vast excess of homoduplex DNA.

In the second experiment, the stability of G/T-mismatched DNA substrate-bound hMSH2/hMSH6 complex was assessed by exposing the mismatched substrate-bound complex to ATP and observing the intensities of S shifted and NS shifted electrophoretic bands at selected times (Figure 8A). A gradual decrease in the intensity of the S shifted electrophoretic band was observed, the band having a half life

of about twenty seconds. Concurrently with the decrease in the intensity of the S shifted electrophoretic band, a gradual but not quantitative increase in the intensity of the NS-shifted electrophoretic band was observed. This observation indicated that ATP induced a time-dependent reduction of specific binding of the hMSH2/hMSH6 complex to the mismatched DNA substrate and that at least a portion of the complex reassociated with the mismatched DNA substrate in a non-specific manner. However, this experiment did not distinguish between the tracking/sliding or simple dissociation and reassociation mechanisms.

In order to distinguish between tracking/sliding and simple dissociation and reassociation, a third experiment was performed. In this experiment, the stability of G/T-mismatched DNA substrate-bound hMSH2/hMSH6 complex was assessed by exposing the mismatched substrate-bound complex to both ATP and a 400-fold excess of non-labeled homoduplex DNA and observing the intensities of S shifted and NS shifted electrophoretic bands at selected times (Figure 8B). As in the second experiment, a gradual decrease in the intensity of the S shifted electrophoretic band was observed, the half-life of the band again being about twenty seconds. This observation was consistent with ATP induction of dissociation of the complex from the mismatched DNA substrate. However, under these conditions, no increase in the intensity of the NS electrophoretic band was observed. Together, these observations indicate that in the presence of excess non-labeled homoduplex DNA, the dissociation of the complex from mismatched DNA does not proceed through the product corresponding to the NS electrophoretic band, but instead is instantaneous and irreversible. When excess non-labeled homoduplex DNA was added to the homologous 81-base pair DNA substrate, the NS electrophoretic band associated with the product formed by contacting the complex with DNA substrate (as in Figure 2B) could be detected (Figure 8D). This observation indicated that, even at 4°C, the product corresponding to the NS band was exceedingly unstable and that level of hMSH2/hMSH6 which remained associated with the DNA substrate was less than the lower limit of accurate quantitation using gel shift analysis.

Without intending to be bound to any particular theory or mechanism, it was concluded that a mechanism involving translocation or treadmilling by the hMSH2/hMSH6 protein complex is an unlikely explanation for the association and dissociation of the complex and mismatched DNA for three primary reasons. First, no intermediate (i.e. no NS shifted electrophoretic band) was observed during ATP-induced dissociation of the complex from the G/T-mismatched DNA substrate in the presence of an excess of unlabeled homologous DNA. Second, dissociation of the hMSH2/hMSH6 complex was too rapid to measure, even at low temperatures. Third, ATP hydrolysis was not required for any of the dissociation processes, as evidenced by the fact that these three experiments were performed in the absence of magnesium. Qualitatively identical results were obtained in the presence of magnesium. Together, the results of these three experiments are consistent with the idea that the release of the hMSH2/hMSH6 protein complex from a mismatched DNA occurs rapidly by a process of simple dissociation.

It is noted that a translocation mechanism involving binding of ATP followed by rapid unidimensional translocation of the complex a distance of at least 40 nucleotides in the absence of hydrolysis cannot be eliminated as a possible mechanism for the association and dissociation of the complex with mismatched DNA. Although this type of mechanism is unlikely, it is possible to visualize a process in which a step-translocation is followed by ATP hydrolysis, displacement of ADP by ATP, and another step-translocation. However, it is worth noting that steady-state ATPase activity of the hMSH2/hMSH6 protein complex is diminished in the presence of duplex DNA, relative to the steady-state ATPase activity of the complex in the presence of mismatched DNA. It is difficult to reconcile the opposing activities of an ATP-bound step-translocation away from the mismatch followed by an ADP-bound mismatch binding stage, into a simple model for mismatch repair.

The hMSH2/hMSH6 protein complex acts as a molecular switch in mismatch recognition

The discovery that the hMSH2/hMSH6 complex is a novel molecular switch which is activatable by ADP was made by reconciling numerous observations described herein. These observations are summarized as follows. ADP and ATP have opposing effects on the role of the hMSH2/hMSH6 complex in mismatched DNA binding. Release of mismatched DNA by the hMSH2/hMSH6 complex is not dependent upon ATP hydrolysis. Hydrolysis of ATP by the hMSH2/hMSH6 complex results in recovery of the mismatch binding ability of the complex. γ-Phosphate hydrolysis is not the rate limiting step of ATPase activity catalyzed by the hMSH2/hMSH6 complex. Displacement of ADP by ATP is the rate limiting step of ATPase activity catalyzed by the hMSH2/hMSH6 complex. Displacement of ADP from the hMSH2/hMSH6 complex by ATP is accelerated in the presence of mismatched DNA, but hydrolysis of the γ-phosphate bond is not accelerated. ATP-dependent release of mismatched DNA from the hMSH2/hMSH6 complex occurs rapidly and by simple dissociation. These observations indicate that γ-phosphate hydrolysis and displacement of ADP by ATP determine whether the hMSH2/hMSH6 complex binds to or is released from mismatched DNA (Figure 9). Recognition of the hMSH2/hMSH6 complex as a molecular switch supports the conclusion that it is a trigger for determining the timing of subsequent excision repair-related events.

Implications for mismatch repair

The number of hMSH2/hMSH6 complex molecules in the nucleus of a proliferating cell has been estimated to exceed one thousand (Drummond et al., 1995, Science 268:1909-1912; Wilson et al., 1995, Cancer Research 55:5146-5150; Meyers et al., 1997, Cancer Res. 57:206-208). The calculated K_d of about 20 nanomolar for mismatched DNA implies that a single mismatched nucleotide in a human cell is likely to be efficiently recognized and bound with high affinity by the hMSH2/hMSH6 complex. In the presence of ADP, this high affinity binding is nearly irreversible. Thus, dissociating the complex from the mismatched DNA in order to allow a

subsequent excision repair event to proceed may be more difficult than binding the complex to the mismatch.

Without wishing to be bound by any particular theory or mechanism of operation, it is proposed that the tight binding of the complex to mismatched DNA acts as a flag for the assembly or nearby localization of cellular excision repair proteins or other factors. When the complete excision repair machinery is assembled nearby, displacement of ADP by ATP could be triggered by a component of the machinery, causing dissociation of the complex from the mismatched region. Exonuclease excision and replacement of the mismatched region may then proceed.

Once released from the mismatched nucleotides, the intrinsic ATPase activity of the hMSH2/hMSH6 complex could cause hydrolysis of the ATP molecule which displaced ADP, yielding the ADP-bound form of the complex that is competent for mismatch binding. As a free protein complex, hMSH2/hMSH6 does not efficiently exchange ADP bound thereto, providing a long-term mismatch recognition-competent molecule.

The enhanced ability ATP to displace ADP bound to the hMSH2/hMSH6 complex in the presence of mismatched DNA may be explained as follows. It is possible that the hMSH2/hMSH6 complex oscillates between a mismatch recognition-competent (i.e. ADP-bound) form and a mismatch-recognition-incompetent (i.e. ATP-bound) form. Such oscillation seems energetically wasteful and unlikely to occur *in vivo*. Concern for the apparent energetic wastefulness of such oscillation may somewhat diminished by comparing the value of k_{cat} for robust ATPases (i.e. 100-1000 minute^{-1}; Graves Woodward et al., 1996, J. Biol. Chem. 271:13629-13635; Jiang et al., 1997, J. Biol. Chem. 272:7626-7632; Wong et al., 1996, Biochemistry 35:5726-5734)) to the value of k_{cat} for the hMSH2/hMSH6 complex in the presence of the G/T-mismatched DNA substrate (i.e. 26 minute^{-1}). Clearly, the hMSH2/hMSH6 complex would hydrolyze less ATP than robust ATPases, under the conditions described herein.

There are at least two other explanations of the physiological relevance of the enhanced ability of ATP to displace ADP bound to the complex. First, although the displacement rate is clearly faster in the presence of the G/T-mismatched DNA substrate than it is in the absence of DNA, the rate is still slow relative to the rate of mismatch repair events. Thus, the complex is able to complete few, if any, cycles of association with and dissociation from mismatched DNA and, as a result, hydrolyzes few, if any, ATP molecules. Alternately, the ADP-bound form of the complex when bound to mismatched DNA may be stabilized by other proteins that provide the ultimate trigger for displacement of complex-bound ADP by ATP during the course of a mismatch repair event. Regardless of the mechanism involved, it is clear from the observations presented in this Example that binding of the hMSH2/hMSH6 complex to the G/T-mismatched DNA substrate elicited a change in the protein such that, upon binding, the complex became competent to exchange ADP for ATP. In the absence of DNA, the complex could not exchange ADP for ATP.

Generality of MutS function

The studies described in this Example, which involved the human mismatch binding reaction catalyzed by the hMSH2/hMSH6 protein complex, are consistent with genetic studies performed in both bacteria and yeast. In those studies, mutation of the adenine nucleotide binding and hydrolysis domain(s) resulted in a dominant mutator phenotype (Haber et al., 1991, EMBO. J. 10:2707-2715; Wu et al., 1994, J. Bacteriol 176:5393-5400; Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447). Those studies, combined with the studies described in this Example, indicate that there may be two opposing functional alterations of MutS homologs that can cause such a dominant mutator phenotype. First, alteration of the ability of the homolog to bind and/or exchange ADP for ATP can cause a dominant mutator phenotype. Second, alteration of the ability of the homolog to hydrolyze ATP can similarly cause such a phenotype. Inability of the homolog to bind to ADP or to exchange ADP for ATP would result in a permanently mismatched DNA-bound form of the MutS homolog. This form of the homolog would exclude the repair machinery from the mismatch site.

Inability of the MutS homolog to hydrolyze ATP would result in a form of the homolog that would be unable to bind to mismatched DNA and which, therefore, would be unable to recruit the cellular mismatch repair proteins and factors to the site of the mismatch. Each these conditions would cause an increased mutation rate in the organism containing the homolog, as a consequence of the organism's depressed ability to repair mismatched DNA (Wu et al., 1994, J. Bacteriol 176:5393-5400).

Preliminary studies performed using the methods described herein and using purified *Escherichia coli* MutS protein suggest that *E. coli* MutS also functions as a molecular switch, albeit with a more stringent requirement for mismatch-induced nucleotide exchange. Therefore, the properties of the MutS homologs hMSH2 and hMSH6, as described herein appear to be properties of all MutS homologs, including, but not limited to, *E. coli* MutS, the human homologs hMSH2, hMSH3, and hMSH6.

Similarity of the hMSH2/hMSH6 complex to G-protein switches

The hMSH2/hMSH6 molecular switch is, in some respects, similar to G-protein switches which have been described (Bokoch et al., 1993, FASEB J. 7:750-759). G-proteins are known to trigger translocation events associated with protein synthesis (Laalami et al., 1996, Biochimie 78:577-589; Parmeggiani et al., 1981, Mol. Cell Biochem. 35:129-158), cascade events associated with cell signaling (Mederna et al., 1993, Crit. Rev. Oncol. 4:615-661; Wiesmuller et al., 1994, Cell Signal 6:247-267) and physiological responses to ligand-binding by membrane receptors (Spiegel, 1987, Mol. Cell Endocrinol. 49:1-16). Many G-proteins are associated with regulators that stimulate both the GTPase activity of the G-protein (Tocque et al., 1997, Cell Signal 9:153-158) and the exchange of G-protein-bound GDP for GTP (Dohlman et al., 1997, J. Biol. Chem. 2 72:3871-3874; Quilliam et al., 1995, Bioessays 17:395-404). In fact, the Ras G-protein was determined to be unable to catalyze GTP hydrolysis because it is unable to exchange GDP for GTP. The discovery of a GTPase activating protein (GAP) that stimulated GTP γ-phosphate hydrolysis, and a guanine nucleotide exchange factor (GNEF) that stimulated the exchange of GDP for GTP, provided a model for

regulation of the Ras G-protein switch (Tocque et al., 1997, Cell Signal 9:153-158; Dohlman et al., 1997, J. Biol. Chem. 2 72:3871-3874).

It has therefore been discovered that protein regulation of the excision-resynthesis processes associated with mismatch repair occurs by stimulation of the ATPase activity of the hMSH2/hMSH6 complex or of the ability of the complex to exchange ADP for ATP. The latter stimulation can occur either by stabilizing the ADP-bound form of the complex or by stimulating exchange of ADP for ATP to effect release of the complex from mismatched DNA. The human MutL homologs, hMLH1 performs these regulatory functions.

Example 2. A Mouse Construct Nullizygous for both the *msh2* and *p53* Genes and Methods of Making and Use Thereof

Transgenic mice which are nullizygous for both the *Msh2* gene and the *p53* gene have been made, and are referred to herein as $Msh2^{-/-}p53^{-/-}$ mice. Other transgenic animals which are nullizygous for both the *Msh2* gene and the *p53* gene, and which particularly include mammals, especially including rodents such as mice and rats, may be made using methods analogous to those described herein and are useful in the screening methods described herein.

The development of female $Msh2^{-/-}p53^{-/-}$ mouse embryos is phenotypically arrested at approximately the 9.5 day stage, and apoptosis is induced shortly thereafter in the cells of these embryos. Male $Msh2^{-/-}p53^{-/-}$ mouse embryos are viable, but succumb to tumors significantly earlier than either $Msh2^{-/-}$ or $p53^{-/-}$ littermates (i.e. nullizygous *Msh2* mice or nullizygous *p53* mice, respectively). Furthermore, the frequency of microsatellite instability (MSI) in tumor tissue obtained from $Msh2^{-/-}p53^{-/-}$ mice is not significantly different than the frequency in tumor tissue obtained from $Msh2^{-/-}$ mice. Synergism in tumorigenesis and independent segregation of the MSI phenotype suggest that *Msh2* and *p53* are not genetically epistatic.

$Msh2^{-/-}p53^{-/-}$ mice are useful as models of disease or disorder states which cannot be identified in mice nullizygous for only one of the *Msh2* gene or the

p53 gene. Furthermore, *Msh2*$^{-/-}$*p53*$^{-/-}$ mice are useful for identifying compositions which affect the onset or progression of such a disease or disorder state. Thus, a *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse is particularly useful as a model system for studying multistep tumorigenesis.

As used herein, the term "nullizygous" refers to an animal which possesses a pair of null mutant alleles at a given genetic locus. Hence, a nullizygous *Xxx* mouse (wherein *Xxx* is any gene normally present in a mouse) does not possess a functional *Xxx* gene, whereas a wild-type mouse may possess one or two functional copies of the *Xxx* gene. To illustrate the notation used herein, the term "nullizygous *Xxx* mouse" is synonymous with the term "*Xxx*$^{-/-}$ mouse." Similarly, a "heterozygous *Xxx* mouse" has one functional *Xxx* allele and one non-functional *Xxx* allele, and is synonymous with the term "*Xxx*$^{+/-}$ mouse." A "wild type mouse" has at least one copy, and possibly two copies, of a functional *Xxx* allele, and is synonymous with the term "*Xxx*$^{+/\pm}$ mouse." A "homologous wild type mouse' has two copies of a functional *Xxx* allele, and is synonymous with the term "*Xxx*$^{+/+}$ mouse."

The materials and methods used in the experiments presented in this Example are now described.

Generation of *Msh2*$^{-/-}$*p53*$^{-/-}$ Mice

Methods for making heterozygous and nullizygous *Msh2* mice and heterozygous and nullizygous *p53* mice have been described (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nat. Genet. 11:64-70; Donehower et al., 1992, Nature 356:215-221; Jacks et al., 1994, Curr. Biol. 4:1-7; Purdie et al., 1994, Oncogene 9:603-609).

Mice heterozygous for the *Msh2* gene (i.e. *Msh2*$^{+/-}$*p53*$^{+/+}$ mice) on a mixed C57BL/6J and 129/Ola background and mice heterozygous for the *p53* gene (i.e. *Msh2*$^{+/+}$*p53*$^{+/-}$ mice) on a mixed C57BL/6J and 129/Sv were mated to produce F1 progeny heterozygous for both genes (i.e. *Msh2*$^{+/-}$*p53*$^{+/-}$ mice). Heterozygous sibling F1 progeny were intercrossed to produce progeny nullizygous for both *Msh2* and *p53*

(i.e. *Msh2*$^{-/-}$*p53*$^{-/-}$ mice). Mice were genotyped using *Msh2*- and *p53*- specific PCR-based assays, using methods well known in the art.

Isolation of Genomic DNA

Mouse genomic DNA was extracted from ear-notched tissue of mice and from amniotic tissue of mouse embryos at 9.5, 11.5, or 13.5 days of development, using a QIAamp Tissue Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

PCR-based Genotyping of Mice

A three-primer assay specific for *Msh2* was carried out as described (Reitmair et al., 1995, Nat. Genet. 11:64-70). A four-primer assay specific for *p53* was carried out using 50 ng of template DNA in a 50 µl reaction mixture containing 1 unit of *Taq* polymerase (Fisher Scientific, Malvern, PA) and 100 mM each of the following primers, each of which is identified with a five digit number and the sequence of each of which is listed:

10681 (5'-GTGTTTCATTAGTTCCCCACCTTGAC-3');
10480 (5'-ATGGGAGGCTGCCAGTCCTAACCC-3');
10588 (5'-GTGGGAGGGACAAAAGTTCGAGGCC-3'); and
10930 (5'-TTTACGGAGCCCTGGCGCTCGATGT-3').

The amplification reaction involved 35 cycles of amplification (94°C, 15 seconds; 56°C, 30 seconds; 72°C, 1 minute) using a Perkin-Elmer GeneAmp 9600 thermal cycler. The wild-type primers, *10681* and *10480*, amplified a product of about 320 bp length, and the targeted allele (i.e. *p53*$^{-}$) primers, *10588* and *10930*, amplified a product of about 150 bp length.

The gender of embryos was determined using primers specific for the Y-chromosome gene as described (Sah et al., 1995, Nat. Genet. 10:175-180). The presence of the X-chromosome was confirmed separately in all cases using the following two X-chromosome specific primers to amplify the locus *DXMIT6*:
5'-ACCATTCAAATTGGCAAGG-3'; and
5'-GTGGCTCGAGTTGTTTGCAG-3'.

PCR cycling conditions were as described above for *p53* genotyping, except that the annealing temperature was 53°C, rather than 56°C. The X-chromosome specific primers amplified a product of about 210 bp in length. All PCR amplification products were resolved by electrophoresis on a 2% (w/v) agarose gel alongside a 100 bp polynucleotide ladder and were visualized by ethidium bromide staining.

Timed Pregnancies

Male and female mice that were $Msh2^{+/-}p53^{+/-}$, $Msh2^{+/-}p53^{-/-}$, or $Msh2^{-/-}p53^{+/-}$ were mated and each of the females was examined daily for the presence of a vaginal plug (an indicator of pregnancy which appears at about day 0.5 of embryo development). Pregnant females were sacrificed at 13.5 days, at 11.5 days, or at 9.5 days gestation. Embryos were dissected out from the pregnant females into Hank's Balanced Salt Solution (Gibco BRL, Grand Island, NY) under a dissecting microscope, fixed in 4% buffered formalin, and documented by photomicrography. Amnion was retrieved from each embryo, DNA extracted therefrom, and the sex and genotype of each embryo determined by PCR.

Histology

Tissue specimens were fixed in 10% (v/v) or 4% (v/v) buffered formalin and embedded in paraffin. Histological analysis was carried out on 3 micrometer-thick sections stained with hematoxylin and eosin (H&E).

TUNEL Assay

Paraffin-embedded tissue sections were dewaxed and rehydrated through a graded alcohol series, using methods well known in the art. Apoptotic cells and appropriate positive and negative control samples were analyzed using the *In Situ* Cell Detection Kit, AP with NBT/BCIP, manufactured by Boehringer Mannheim (Indianapolis, IN), according to the manufacturer's instructions. TUNEL-stained tissue sections were analyzed both by fluorescence and light microscopy.

Kaplan-Meier Survival

Kaplan-Meier survival probability was calculated for mice that were found dead or were sacrificed when found to be moribund. The age of the mice was

calculated in days. Because no mice died in the control group, confidence limits could not be determined.

Microsatellite Instability in Lymphoid Tumors

Paired ear-notch (i.e. normal) and lymphoid tumor tissues were analyzed for microsatellite instability at five chromosomal loci: *D17Mit123*, *D10Mit2*, *D6Mit59*, *D4Mit27*, and *D3Mit203*. Microsatellite primer sequence pairs appropriate for amplification of these loci were obtained from the World Wide Web site of the Whitehead Institute for Genome Research (http://www.genome.wi.mit.edu), and were chosen to amplify fragments containing at least twenty dinucleotide repeat sequences. PCR amplifications were carried out in a total reaction volume of 25 µl, using 50 ng of DNA as template, 100 mM of each primer pair and 1 unit of *Taq* polymerase (Fisher). The amplification reaction involved 35 cycles of amplification (94°C, 15 seconds; 56°C, 30 seconds; 72°C, 1 minute). Amplified products were resolved by electrophoresis on a 6.7% (w/v) denaturing polyacrylamide gel and were visualized by silver nitrate staining of the gel.

The results of the studies described herein are now described.

Twenty-one $Msh2^{-/-}p53^{-/-}$ mice were generated from $Msh2^{+/-}p53^{+/-}$, $Msh2^{-/-}p53^{+/-}$, or $Msh2^{+/-}p53^{-/-}$ parents. When the gender of each of the twenty-one $Msh2^{-/-}p53^{-/-}$ mice was examined, all were determined to be male $Msh2^{-/-}p53^{-/-}$ mice. The absence of female $Msh2^{-/-}p53^{-/-}$ offspring is highly significant ($p < 0.001$) and is unlikely to reflect the intrinsic bias for males observed in the colony corresponding to the mice, wherein the normal male:female ratio is 181:138.

The fertility of male $Msh2^{-/-}p53^{-/-}$ mice could not be determined, because they succumbed to tumors before they successfully mated. However, pathological examination of the testes of the male $Msh2^{-/-}p53^{-/-}$ mice did not reveal gross abnormalities upon autopsy, and histology revealed mature spermatogenesis in all twenty-one of the male $Msh2^{-/-}p53^{-/-}$ mice. Taken together, these results suggest that $Msh2^{-/-}p53^{-/-}$ male mice are not likely to be sterile.

No gross morphological abnormalities were observed in *Msh2*$^{-/-}$ animals either *in utero* or post-natally (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nat. Genet. 11:64-70). In addition, the number of male and female *Msh2*$^{-/-}$ mice in the studies described herein was in accordance with the expected 1:1 ratio, which suggests that male and female nullizygous *Msh2* mice are equally viable. However, a decrease in the number of live born nullizygous *p53* mice from the expected Mendelian ratio was observed, which is qualitatively similar to previous reports, although our limited numbers did not indicate a sex bias (Sah et al., 1995, Nat. Genet. 10:175-180; Nicols et al., 1995, Nat. Genet. 10:181-187).

No female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice were found at weaning and none of thirteen one-day-old pups which were found dead in the litters of mating pairs were *Msh2*$^{-/-}$ *p53*$^{-/-}$. Thus, all female embryos nullizygous for both *Msh2* and *p53* died *in utero*. To determine the point in embryonic development at which these embryos died, numerous timed pregnancies were established. Because *Msh2*$^{-/-}$*p53*$^{-/-}$ males were not available and *Msh2*$^{-/-}$*p53*$^{-/-}$ females were not viable, pairs of mice, each of which mice was a *Msh2*$^{+/-}$*p53*$^{+/-}$, *Msh2*$^{+/-}$*p53*$^{-/-}$, or *Msh2*$^{-/-}$*p53*$^{+/-}$ mouse, were mated to produce *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos. Pregnant females were sacrificed at 9.5, 11.5, and 13.5 days of gestation, the embryos were pathologically assessed for developmental defects and their genotype and gender were determined by PCR. The results of these analyses are presented in Table 1. A total of twenty-one embryos and six resorption sites were recovered from three females at day 13.5 of gestation. Of the twenty-one 13.5 day embryos, two male *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos and no female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos were recovered, although a total of five *Msh2*$^{-/-}$*p53*$^{-/-}$embryos were statistically expected. Two 13.5 day embryos (one male *Msh2*$^{+/-}$*p53*$^{-/-}$; one female *Msh2*$^{-/-}$*p53*$^{+/-}$) displayed exencephaly, while all other 13.5 day embryos appeared normal (Sah et al., 1995, Nat. Genet. 10:175-180).

Table 1. Sex and Morphological Phenotype of Timed Post-Implantation Embryos

Days Development	Resorption Sites	# of Embryos	Embryos Typed	Female *Msh2*$^{-/-}$ *p53*$^{-/-}$		Male *Msh2*$^{-/-}$ *p53*$^{-/-}$	
				Nor	Abnr	Nor	Abnr
e9.5	3	30	28	3	1	2	1
e11.5	11	21	17	0	4	2	0
e13.5	6	21	21	0	0	2	0
*28	-	*96	*96	*0	*0	*21	*0

Embryos that arrested in development, that were in resorption, or that displayed gross abnormalities were classified as abnormal (Abnr), while those embryos which were not arrested in development, were not in resorption, and did not display gross abnormalities were classified as normal (Nor). Thirteen newborn pups that were found dead, none of which were *Msh2*$^{-/-}$*p53*$^{-/-}$, are not represented in this Table.

*Refers to live-born animals at twenty-eight days following birth.

Twenty-one embryos and eleven resorption sites were recovered from three pregnant females at day 11.5 of gestation. Of these, complete PCR typing results were determined for seventeen embryos and one resorption site. Five embryos were determined to be *Msh2*$^{-/-}$*p53*$^{-/-}$, although eight *Msh2*$^{-/-}$ *p53*$^{-/-}$ embryos were statistically expected. Two of the five embryos were males that appeared morphologically normal (one is depicted in Fig. 1, Panel A), and three of the five embryos were females, all three of which had undergone developmental arrest, and all three of which are depicted in Fig. 1, Panels B, C, and D. The three female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos appeared opaque and somites were not visible. Based on the gross morphology of the three female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos, it was calculated that they died at 9.5 days of development. The tissue from the resorption site was typed as female *Msh2*$^{-/-}$ *p53*$^{-/-}$.

Thirty embryos and three resorption sites were recovered from pregnant females at day 9.5 of gestation. Twenty-eight embryos and one resorption site were successfully typed. Two embryos and a resorption site were found to be male *Msh2*$^{-/-}$*p53*$^{-/-}$, and four embryos were typed as female *Msh2*$^{-/-}$*p53*$^{-/-}$. Six *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos were statistically expected. Neither of the male *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos exhibited any gross morphological abnormality. It is likely that the male *Msh2*$^{-/-}$*p53*$^{-/-}$ resorption site represents a spontaneous abortion event. In one of the four female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos, the anterior neural tube was not closed and the heart was not seen to beat, which should occur around day 9 of development. These observations are consistent with a developmental delay that could result from late fertilization or implantation or alternatively, from a developmental abnormality that is apparent at day 9.5.

Paraffin embedded tissue sections from wildtype and *Msh2*$^{-/-}$ *p53*$^{-/-}$ female embryos, as depicted in Fig. 2, from *Msh2*$^{-/-}$ embryos, and from *p53*$^{-/-}$ embryos were examined at day 11.5 and at day 13.5. While the wildtype, *Msh2*$^{-/-}$, and *p53*$^{-/-}$ embryos had clearly distinguished developmental features at day 11.5, the arrested *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryos contained noncohesive cells without preservation of embryonal tissue structures. In addition, H&E stained *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryonic tissue sections appeared to contain an large number of "blebbed" structures typical of apoptotic cells. Furthermore, loss of nuclear hematoxylin stain typical for necrosis was not observed in H&E stained *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryonic tissue sections (Fig. 2, Panel B).

TUNEL staining was performed on the paraffin embedded tissue sections (Fig. 2, Panels C-F). Although wildtype (Fig. 2, Panels C and E), *Msh2*$^{-/-}$, and *p53*$^{-/-}$ embryos displayed circumscribed foci of apoptotic cells characteristic of normal embryonal development, *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryos displayed global catastrophic apoptosis (Fig. 2, Panels D and F).

Furthermore, fluorescence TUNEL staining of *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryos revealed a speckled intracellular patterning characteristic of fragmented chromatin (Fig. 2, Panel F). It was estimated that between about 60% and about 90% of cells in *Msh2*$^{-/-}$*p53*$^{-/-}$ female embryos were undergoing visible apoptosis, as assessed by H&E and TUNEL staining.

Kaplan-Meier survival analysis was performed on a cohort of ninety-six mice, the data for which analysis are graphically depicted in Fig. 3. *Msh2*$^{-/-}$*p53*$^{-/-}$ mice began to die of generalized lymphomas at day 53 after birth and all twenty-one *Msh2*$^{-/-}$*p53*$^{-/-}$ mice were dead within four months of birth. In contrast, only 18% (eight of forty-four) of *Msh2*$^{-/-}$ littermates and 71% (five of seven) of *p53*$^{-/-}$ littermates were dead at the time the mice were analyzed. Thus, *Msh2*$^{-/-}$*p53*$^{-/-}$ mice had a significantly ($p<0.001$) reduced median survival time of 73 days compared with the median survival time of either *Msh2*$^{-/-}$ mice (i.e. 200 days) or *p53*$^{-/-}$ mice (i.e. 149 days). Furthermore, all twenty-four wild-type (i.e. *Msh2*$^{+/\pm}$*p53*$^{+/\pm}$) littermates were alive after approximately ten months. These results indicate that *Msh2* and *p53* null mutations cooperatively promote tumorigenesis. *p53* has also been shown to cooperate with a variety of other genes in mouse tumorigenesis models (Blyth et al., 1995, Oncogene 10:1717-1723; Williams et al., 1994, Cold Spring Harbor Symp. Quant. Biol. 59:449-457; Williams et al., 1994, Cell 79:329-339; Donehower et al., 1995, Genes Dev. 9:882-895; Nacht et al., 1996, Genes Dev. 10:2055-2066). However, as is apparent from Fig. 3, the effect on tumor-related death of having dual null mutations of *Msh2* and *p53* is greater than the sum of the effects of having a single null mutation in *Msh2* or *p53* alone. Thus, the *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse described herein has a phenotype which is significantly different from a mere combination of the phenotype of a *Msh2*$^{-/-}$ mouse and the phenotype of a *p53*$^{-/-}$ mouse.

Pathological examination of tumors showed that all twenty-one *Msh2*$^{-/-}$*p53*$^{-/-}$ mice developed highly aggressive generalized lymphomas

involving major organs. In addition, a pleomorphic sarcoma in the flank, a malignant fibrous histiocytoma of the neck, and a tubular adenoma of the small intestine were observed, while other epithelial neoplasms were not detected. The tumor spectrum of *Msh2*$^{-/-}$ and *p53*$^{-/-}$ mice appeared similar to previous observations (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nat. Genet. 11:64-70; Donehower et al., 1992, Nature 356:215-221; Jacks et al., 1994, Curr. Biol. 4:1-7; Purdie et al., 1994, Oncogene 9:603-609). The tumor spectrum of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice differs significantly from the tumor spectrum of either *Msh2*$^{-/-}$ or *p53*$^{-/-}$ mice. Thus, *Msh2*$^{-/-}$*p53*$^{-/-}$ mice have utility different from that of either *Msh2*$^{-/-}$ or *p53*$^{-/-}$ mice.

Normal and tumor tissues obtained from individual *Msh2*$^{-/-}$*p53*$^{-/-}$ mice were examined for microsatellite instability at five loci: *D17Mit123*, *D10Mit2*, *D6Mit59*, *D4Mit27*, and *D3Mit203* (Fig. 4). The results of these MSI studies are presented in Table 2. The frequency of MSI in tumor tissues obtained from *Msh2*$^{-/-}$ mice was not significantly different ($p>0.05$) the frequency of MSI in tumor tissues obtained from *Msh2*$^{-/-}$*p53*$^{-/-}$ mice. Microsatellite instability was not observed in lymphomatous tumor tissue obtained from the seven *p53*$^{-/-}$ mice examined. The observation that *Msh2*$^{-/-}$*p53*$^{-/-}$ mice developed earlier onset of tumor-related disease, combined with the observed separate segregation of the MSI phenotype with the *Msh2* allele, suggests that *Msh2* and *p53* are not genetically epistatic.

Table 2. The Frequency of Microsatellite Instability in *p53*$^{-/-}$, *Msh2*$^{-/-}$, and *Msh2*$^{-/-}$ *p53*$^{-/-}$ Mice.

Genotype Tumor / Normal Pairs	Tumors Examined (n)	MSI at ≥1 Locus	MSI at ≥2 Loci	MSI at ≥3 Loci
p53$^{-/-}$	7	0 (0%)	0 (0%)	0 (0%)
Msh2$^{-/-}$	8	6 (75%)	4 (50%)	3 (38%)
**Msh2*$^{-/-}$ *p53*$^{-/-}$	21	17 (81%)	14 (67%)	12 (57%)

*Because female *Msh2*$^{-/-}$ *p53*$^{-/-}$ mice died during embryonic development, this refers to only male *Msh2*$^{-/-}$ *p53*$^{-/-}$ mice.

It is remarkable that female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos underwent global developmental arrest and that widespread apoptosis of the cells of such embryos occurred around day 9.5 of development. That these embryos underwent implantation and gastrulation strongly suggests that they are capable of executing the earlier stages of embryogenesis. The arrested phenotype is reminiscent of that described for a small proportion of female *p53*$^{-/-}$ mice (Sah et al., 1995, Nat. Genet. 10:175-180). However, unlike *p53*$^{-/-}$ mice, no normal female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice or embryos were observed beyond 9.5 days of embryonic development. This observation supports the conclusion that the female embryonic lethality of *Msh2*$^{-/-}$*p53*$^{-/-}$ mice is highly penetrant. In addition, none of the female *Msh2*$^{-/-}$*p53*$^{-/-}$ embryos displayed the exencephaly that characterized the *p53*$^{-/-}$ mice (Sah et al., 1995, Nat. Genet. 10:175-180). Furthermore, while there was no difference in apoptosis observed in developing *p53*$^{-/-}$ mouse embryos, global catastrophic apoptosis was clearly observed in all the *Msh2*$^{-/-}$*p53*$^{-/-}$ female mouse embryos examined. These results suggest that female *Msh2*$^{-/-}$*p53*$^{-/-}$ mice succumb at an earlier stage and by an entirely different pathology than *p53*$^{-/-}$ mice.

Without being bound to any particular theory, the lethality observed in female *Msh2*$^{-/-}$*p53*$^{-/-}$ mouse embryos is consistent with the following explanation. In the female embryonic lineage, dosage compensation

is achieved by random X chromosome inactivation around the time of gastrulation, at which time intense embryonic cellular proliferation and apoptosis promote embryonic differentiation (Lyon, 1961, Nature 190:372-373; Rastan, 1994, Curr. Opin. Genet. Dev. 4:292-297; Theiler, 1972, In: The House Mouse Development and Normal Stages from Fertilization to 4 Weeks of Age, Springer-Verlag, New York, p. 168). The global apoptotic effect need not occur coincidentally with X chromosome inactivation. The full effect of dysregulation may only become apparent after a number of cell divisions when the embryo undergoes a further burst of proliferation during embryonic 'turning' between 8 and 9.5 days.

It has been shown that the inactivated X chromosome replicates late in S phase (Taylor, 1960, J. Biophys. Biochem. Cytol. 7:455-464; Tagaki, 1974, Exp. Cell Res. 86:127-135). In addition, cells deficient in *p53* have been shown to be defective for damage-induced G_1/S checkpoint arrest, and cells that are deficient in MMR have been shown to be deficient for damage-induced G_2/M checkpoint arrest (Baker et al., 1990, Science 249:912-915; Diller et al., 1990, Mol. Cell. Biol. 10:5772-5781; Lin et al., 1992, Proc. Natl. Acad. Sci. USA 89:9210-9214; Hawn et al., 1995, Canc. Res. 55:3721-3725; Marra et al., 1996, Oncogene 13:2189-2196). Thus, female-specific *Msh2*$^{-/-}$*p53*$^{-/-}$ embryo lethality may result from dysregulation of damage-induced arrest checkpoint control, wherein such dysregulation is caused by a deficiency of both *p53* and *Msh2*, and whereby such dysregulation results in an inability of *Msh2*$^{-/-}$*p53*$^{-/-}$ cells to arrest cell division and repair damage introduced into the late replicating inactive X chromosome. Such damage could take the form of nonreplicated regions or chromosomal fragments that have resulted from inappropriate cell division prior to the completion of inactive X chromosome replication. Fragmented, reactivated, or otherwise altered inactive X chromosomes may then lead to global catastrophic cellular failure, developmental arrest, and apoptosis. Furthermore, the observation that the

highest levels of p53 mRNA are detected in wild-type embryos between 9 and 11 days of development suggests an important role for p53 protein within this time frame (Rogel et al., 1985, Mol. Cell. Biol. 5:2851-2855).

Screening Methods

A standard screening procedure is now described which is useful for determining the tumorigenetic potential of a compound. *Msh2*$^{-/-}$ *p53*$^{-/-}$ mice are generated as described herein. While this procedures is described with respect to particular protocols and mice, it will be appreciated that the screening procedure described should not be construed to limit the invention in any way.

A predetermined amount of the compound is administered to the *Msh2*$^{-/-}$ *p53*$^{-/-}$ mouse by any practical means. The method of administration of the compound is not critical. By way of example, the compound may be administered orally, intraperitoneally, intravenously, topically, intramuscularly, or via a pulmonary route.

To reduce any potential for bias, the study is blinded. A first investigator treats all mice with compound(s) and identifiably marks or cages the transgenic mice, so that the nature of the treatments will not be known to a second investigator, who performs all tumor counts, weighing, and general observations.

Following administration of the compound, the *Msh2*$^{-/-}$ *p53*$^{-/-}$ mouse, each *Msh2*$^{-/-}$ *p53*$^{-/-}$ mouse is observed for about four months. Each mouse is examined approximately daily. Every week, each mouse is weighed, observed for any clinically-relevant symptoms, and the number and extent of tumors are assessed.

After observations are completed, the rate of tumor incidence and the tumor yield are determined for each group of *Msh2*$^{-/-}$ *p53*$^{-/-}$ mice to which the compound was applied. A higher or lower rate of tumor incidence or a higher or lower tumor yield for a group of *Msh2*$^{-/-}$ *p53*$^{-/-}$ mice to which the

compound was applied, compared with the levels of tumor incidence and tumor yield for a group of *Msh2*$^{-/-}$ *p53*$^{-/-}$ mice to which the compound was not applied, is an indication that the compound affects tumorigenesis.

Preferably, groups of *Msh2*$^{-/-}$ *p53*$^{-/-}$ mice are used, with each mouse in a group being treated identically. Also preferred are studies in which one of at least three different dose levels of the compound are applied to the mice in each of at least three corresponding groups of transgenic mice. It is preferred, where possible, to demonstrate a statistically significant difference ($P < 0.05$) between the average rate of tumor incidence or the average tumor yield for the first dose level and the average rate of tumor incidence or the average tumor yield for the third dose level.

Msh2$^{-/-}$ *p53*$^{-/-}$ mice may also be used to identify the ability of a compound to affect apoptosis. This assay is performed identically to the tumorigenesis assay described herein, except that the compound is administered to *Msh2*$^{-/-}$ *p53*$^{-/-}$ mice *in utero*, and the embryonic development of female *Msh2*$^{-/-}$ *p53*$^{-/-}$ mice is assessed, rather than tumor incidence or yield. A difference between embryonic development in female *Msh2*$^{-/-}$ *p53*$^{-/-}$ mouse embryos to which the compound was administered and embryonic development in female *Msh2*$^{-/-}$ *p53*$^{-/-}$ mouse embryos to which the compound was not administered is an indication that the compound affects apoptosis.

Msh2$^{-/-}$ *p53*$^{-/-}$ mice may also be used to identify the ability of a compound to affect the aging process. This assay is performed identically to the apoptosis assay described herein, except that a difference between embryonic development in female *Msh2*$^{-/-}$ *p53*$^{-/-}$ mouse embryos to which the compound was administered and embryonic development in female *Msh2*$^{-/-}$ *p53*$^{-/-}$ mouse embryos to which the compound was not administered is an indication that the compound affects the aging process.

Methods of making a cell line from a cell of a nonhuman animal are well known in the art. A cell line which is nullizygous for both the *Msh2*

gene and the *p53* gene can be made by culturing a cell obtained from the nonhuman animal of the invention.

Example 3

Mismatch repair, molecular switches, and signal transduction

The foundation of molecular switches in biology is grounded in translation elongation and cellular signal transduction. In these systems, guanine nucleotide-bound proteins (G proteins) produce the ON and OFF signaling states that act as gates for downstream biochemical processes. Recent studies on the human mismatch repair reaction have suggested a similar molecular switch that relies on adenine nucleotide-bound forms (A proteins) to produce an ON and OFF signaling state. In the field of signal transduction, the concept of a molecular switch is elementary whereas the biochemical processes of DNA repair appear foreign. Similarly, the field of DNA repair recognizes the complex machinery required for DNA manipulation events but regards biochemical signaling processes as essential cellular input but outside the genome juggernaut. The concept of a molecular switch as an integral step in mismatch repair should accelerate communication between these two fields toward a resolved and unified mechanism for biological signaling processes.

Genetics of mismatch repair

There are at least three ways in which mismatched nucleotides arise in DNA: (1) physical or chemical damage to the DNA and its precursors, such as deamination of 5-methyl-cytosine (Friedberg 1990); (2) misincorporation of nucleotides during DNA replication can yield mismatched base pairs as well as the insertion and deletion of nucleotides (for review, see Kolodner 1996; Modrich 1989, 1997); and (3) genetic recombination produces regions of heteroduplex DNA that may contain mismatched nucleotides when such heteroduplexes result from the pairing of two different parental DNA sequences (Holliday 1964). Mismatched nucleotides produced by each of these mechanisms are known to be repaired by enzyme systems that are both specific and overlapping (Friedberg 1990).

The most extensively studied system for mismatch repair (MMR) is the DNA adenine methylation (Dam)-instructed pathway of *Escherichia coli* (for review, see Modrich 1989; Modrich and Lahue 1996). The Dam-instructed pathway promotes a long-patch (~2 kb) excision repair reaction that is dependent genetically on the *mutH*, *mutL*, *mutS*, and *mutU (uvrD)* gene products. Discrimination of the newly replicated DNA strand from the original template DNA strand is dependent on transient undermethylation of the adenine nucleotide within a GATC Dam sequence. The MutHLS pathway appears to be the most active MMR pathway in *E. coli* and is known to both increase the fidelity of DNA replication as well as to act on recombination intermediates containing mispaired bases (Fishel and Kolodner 1983; Fishel et al. 1986).

Homologs of the prokaryotic MutS and MutL proteins have been identified in nearly every organism with the exception of Archaea (for review, see Kolodner 1996; Fishel and Wilson 1997). At present there are 41 MutS homologs and 21 MutL homologs in the NCBI database. In *Saccharomyces cerevisiae*, six MutS homologs (MSH1–MSH6) and three MutL homologs (MLH1, MLH2, PMS1) have been identified, whereas in human cells a nearly identical set of five MutS homologs (hMSH2–hMSH6) and three MutL homologs (hMLH1, hPMS1, and hPMS2) are known (Fujii and Shimada 1989; Kramer et al. 1989; Linton et al. 1989; Mankovich et al. 1989; Reenan and Kolodner 1992; Fishel et al. 1993; New et al. 1993; Bronner et al. 1994; Burns et al. 1994; Nicolaides et al. 1994; Prolla et al. 1994; Hollingsworth et al. 1995; Palombo et al. 1995; Acharya et al. 1996). Yet, outside of Gram-negative bacteria, there do not appear to be homologs of MutH. Thus, the mechanism of strand discrimination in even close relatives of *E. coli*, the gram-positive bacteria, remains a mystery. The multiple MutS and MutL homologs have been found to participate in the diverse activities of nuclear (MSH2, MSH3, MSH6, MLH1, PMS1) and organellar (MSH1) postreplication mismatch repair as well as distinct meiotic functions (MSH4, MSH5) (Kolodner 1996; Fishel and Wilson 1997).

Biochemistry of mismatch repair

Purification and reconstitution studies by Modrich and colleagues have led to a biochemical model for postreplication mismatch repair in *E. coli*. The reconstituted system requires the MutH, MutL, MutS, and UvrD (he-

licase III proteins along with DNA polymerase III holoenzyme, DNA ligase, single-stranded DNA binding protein (SSB), and one of the single-stranded DNA exonucleases, Exo I, Exo VII, or RecJ (Lu et al. 1983; Su and Modrich 1986; Welsh et al. 1987; Grilley et al. 1989; Lahue et al. 1989; Cooper et al. 1993). In this widely held biochemical model, initiation of a MMR event occurs when MutS recognizes and binds mispaired nucleotides that result from polymerase misincorporation errors (Su and Modrich 1986). It is then suggested that MutS mismatch binding is followed by interaction with the MutL protein (Grilley et al. 1989), which has been proposed to accelerate an ATP-dependent translocation of the MutS–MutL complex (Allen et al. 1997) to a hemimethylated GATC Dam site bound by MutH (Welsh et al. 1987). The MutS–MutL complex then stimulates an intrinsic endonuclease activity of MutH, which results in a specific strand scission on the unmethylated, newly replicated DNA strand (Lahue et al. 1987; Welsh et al. 1987; Cooper et al. 1993). This strand scission directs one of three single-stranded exonucleases (RecJ, Exo I, ExoVII) to degrade the newly replicated strand, which is then resynthesized by the Pol III holoenzyme complex (Lahue et al. 1989). The net result is a strand-specific mismatch repair event that can be bidirectional. Many of the genetic studies performed with this system appear to support this biochemical interpretation. For example, *mutH*, *mutL*, and *mutS* bacteria exhibit a mutator phenotype that is presumed to be the result of the increased probability of misincorporation errors leading to mutations (Demerec et al. 1957; Miyake 1960; Siegel and Bryson 1967; Hill 1970). However, not all predictions arising from this model agree with the genetic results. For example, *recJ exJ exoVII* bacteria do not appear to exhibit a mutator phenotype (Harris et al. 1998), suggesting that there may be other exonuclease(s) or mechanism(s) involved in the mismatch repair process.

Unique functions for the mismatch repair proteins

The most obvious unique function for mismatch repair proteins is the specific mispair binding activity ascribed to MutS homologs (Su and Modrich 1986; Fishel et al. 1994; Chi and Kolodner 1994; Drummond et al. 1995; Acharya et al. 1996; Marsischky et al. 1996; Gradia et al. 1997). A clear function of the MutL homologs has not yet been identified. Classification of MutS and MutL homologs is based on the recognition of highly conserved regions of amino acid identity. The most highly conserved region of the MutS homologs is confined to a region of ~150 amino acids that encompass a helix–turn–helix (HTH) domain associated with a Walker-A adenine nucleotide and magnesium binding motif (Walker et al. 1982). This adenine nucleotide binding domain constitutes 100% of the identity between the known MutS homologs (Fishel and Wilson 1997; Fig. 1). Purified bacterial, yeast, and human MutS homologs have been found to possess an intrinsic low-level ATP hydrolysis (ATPase) activity (Haber and Walker 1991; Chi and Kolodner 1994; Alani et al. 1997; Gradia et al. 1997). This ATPase activity is likely to be important for the function of the MutS homologs because mutation of a conserved lysine residue in the adenine nucleotide binding domain results in a dominant mutator phenotype in both bacteria and yeast (Haber and Walker 1991; Alani et al. 1997). As suggested above, the most widely held model for MMR suggests that MutS mispair binding is followed by MutL association, which then results in an energy-dependent translocation of this complex to a hemimethylated Dam site that is occupied by the MutH protein. In retrospect, this appears to have been a simplistic view because the rate of ATP hydrolysis ($k_{cat} \cong 10\ min^{-1}$) is unlikely to be sufficient to drive mechanical translocation the several hundred to thousand nucleotides (on average) required to encounter a MutH bound hemimethylated site. For example, if one ATP was required to translocate one nucleotide (as the most well-accepted mechanism would suggest), then it would take 25–100 min to encounter a MutH on average. Yet, remethylation of the transiently hemimethylated Dam sites has been found to occur within 0.1–3 min of the passing replication fork (Campbell and Kleckner 1990). Although the ATPase activity could in theory be significantly faster in vivo, no stimulatory factor has been identified to date despite an extensive search. In addition, the prevailing mechanism does not adequately account for MutL function nor the highly conserved domains recognized between MutL homologs from bacteria to man (regions containing 100% identity in 21 homologs).

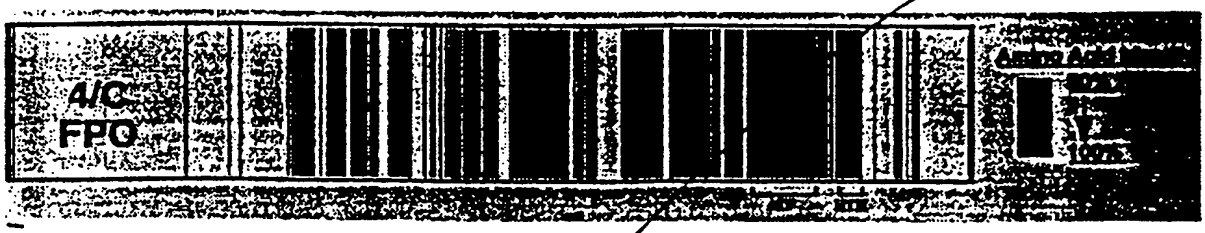


Figure 1. Homology spectrum of 35 MutS homologs. Protein alignment was performed using Megalign (DNASTAR, Madison, WI). Alignments were colorized (DNASTAR, Madison, WI) to indicate 20% (7/35 amino acid identity at an aligned amino acid position), up to 100% (35/35 amino acid identity). The consensus ATP binding domain (ATP) and HTH are shown. The initial alignment included 11 procaryotes and 9 eukaryotes. Whereas the procaryotes generally contribute one MutS homolog, eukaryotes may contribute up to six different MutS homologs (Fishel and Wilson 1997).

The hMSH2–hMSH6 molecular switch

Last year we proposed that the human MutS homologs, hMSH2–hMSH6, function as a molecular switch responsible for the timing of mismatch repair (Gradia et al. 1997; Fig. 2A,B). This hypothesis was based on the observations that: (1) The ADP-bound form has a high affinity for mismatched nucleotides; (2) the exchange of ADP for ATP results in the release from the mismatch in the absence of hydrolysis; (3) release appears to occur by simple dissociation and/or hydrolysis-independent diffusion off the ends of the short oligonucleotides used in these experiments; and (4) hydrolysis of ATP results in recovery of the mismatch-binding competent ADP-bound form. The rate-limiting step and the ultimate control of the hMSH2–hMSH6 molecular switch is likely to be the ADP → ATP exchange, which is exceedingly inefficient in the absence of a mismatched nucleotide. The characteristics of the hMSH2–hMSH6 switch appear most similar to the G-protein mediators of seven-transmembrane (7-TM) domain receptor signaling such as that used by the β-adrenergic and rhodopsin receptors (Fig. 2C) and the prototypical oncoprotein/G-protein Ras (Fig. 2D; Tocque et al. 1997). More specifically, the observation that hMSH2–hMSH6 is induced to exchange ADP for ATP in the presence of a mismatched nucleotide and then dissociates from the mismatch to transduce a signal, mirrors ligand binding by 7-TM receptors, which induce the associated G protein to exchange GDP for GTP and then the GTP-bound form dissociates from the receptor to transduce a signal.

These similarities allowed us to develop two related models for mismatch repair that are fundamentally different from all previously suggested mechanisms. These models are both based on the concept that MutS and its homologs are a novel type of molecular switch that determines the timing and/or appropriate assembly of repair components. The apparent affinity of hMSH2–hMSH6 for mismatched nucleotides ($K_d \cong 2$–20 nM) has suggested that a single mismatch in a human cell should be recognized efficiently and bound. Furthermore, this binding is stabilized slightly in the presence of ADP. We propose two nonexclusive models: In the first model, tight binding to mismatch nucleotides by the ADP-bound form of hMSH2–hMSH6 acts as a flag for the assembly or nearby localization of the excision repair machinery. When the complete system is assembled, then exchange of ADP for ATP would be triggered and hMSH2–hMSH6 would be released from the mismatch (Fig. 2A); thus signaling exonucleolytic excision and resynthesis of the region containing the mismatched nucleotide. Once released from the mismatched nucleotides, the intrinsic ATPase activity of hMSH2–hMSH6 would hydrolyze the bound ATP, resulting in a form that is once again competent for mismatch binding (Fig. 2A). In the second model, recognition of a mismatched nucleotide provokes the ADP → ATP nucleotide exchange and hydrolysis-independent, DNA-associated diffusion of hMSH2–hMSH6 away from the mismatch to the assembled (or partially assembled) mismatch repair components (Fig. 2B). Activation of these components by the confederation of this ATP-bound form of hMSH2–hMSH6 either engages the repair process (signaling the timing of mismatch repair as above) or allows the appropriate assembly of the remaining mismatch repair machinery. This activation event would result in the release of hMSH2–hMSH6, hydrolysis of ATP, and recycling of the mismatch competent form (Fig. 2B). The advantage of this latter model is that the hMSH2–hMS6 remains associated with the DNA in an activated-form poised to transduce the mismatch signal to any nearby mismatch repair components.

As a free protein complex, hMSH2–hMSH6 does not efficiently exchange the ADP remaining after hydrolysis, providing a long-term mismatch-recognition-competent molecule. Again, this ADP-mismatch-bound and ATP-mismatch-released switch appears functionally equiva-

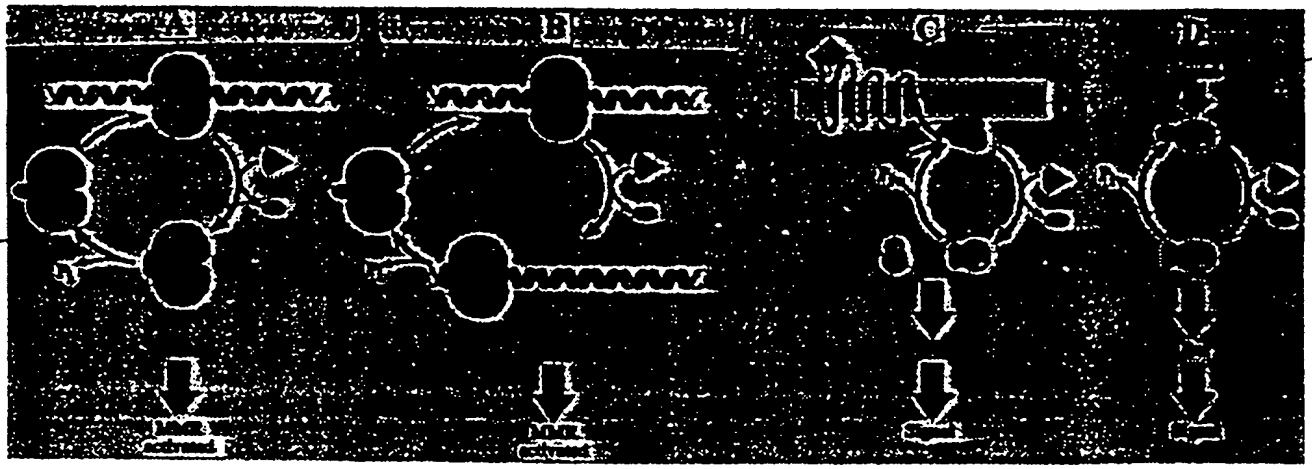


Figure 2. Comparison of the hMSH2–hMSH6 molecular switch with G-protein switches. (*A*) The hMSH2–hMSH6 simple-dissociation molecular switch; (*B*) the hMSH2–hMSH6 hydrolysis-independent translocation molecular switch; (*C*) the heterotrimeric G-protein molecular switch; (*D*) the Ras G-protein molecular switch.

lent to the 7-TM G-protein switches in that a signal is transduced to downstream factors when the NDP is exchanged for the NTP and the G protein dissociates from the 7-TM receptor. The key difference in the mismatch repair models described above and those previously proposed, is the concept that ATP hydrolysis is not required to physically transduce the mismatch binding signal to the downstream machinery, but instead is required to recycle the recognition system. Furthermore, in these models the authentic signal is the conformational transition that ATP binding induces in the MutS homologs hMSH2–hMSH6; thus, the concept of a molecular switch.

Switches in signal transduction

G proteins are the prototypical biological switches (for review, see Sprang 1997 and references therein). Historically, the first recognized G protein was the *E. coli* EF-Tu subunit, which promotes the correct positioning of an aminoacyl-tRNA on an mRNA within the ribosome (Keller and Zamecnik 1955; Nathans et al. 1962). As the process of protein synthesis proceeds, a more classic switch reaction occurs when a second G protein, EF-G, binds to GTP and signals the translocation of a growing peptidyl-tRNA from the A site to the P site, prior to EF-Tu recruitment of the next aminoacyl tRNA. The hydrolysis of the bound GTP by EF-G is used to recycle/reset the system. However, it was the Ras oncogene that focused attention on the function of G proteins (Fig. 2D) (Weinberg 1989). In general, G proteins use the binding energy of GTP to stabilize "switch regions" in conformations that will permit their association with effectors, and the hydrolysis of GTP → GDP + P_i is used to reset the switch. Mutagenesis and X-ray crystallography studies have identified five protein regions (G domains) that contact the GTP at points around the nucleoside rings and along the length of the phosphate chain (Sprang 1997). Two of those G domains include the Walker A and B motifs (Walker et al. 1982). Furthermore, there are at least two switch regions (switch I and II) that undergo conformational transitions in response to nucleotide binding and hydrolysis have been identified in all of the G proteins with known crystal structure (Sprang 1997). Switch regions appear to be involved in making contact with effectors and/or regulators of the GTPase activity. These regions can be extremely malleable and have been shown to be involved in the physical association and dissociation of components in the signaling cascade (Sprang 1997).

One of the most important observations concerning G proteins is their regulation by associated proteins (Bokoch and Der 1993). There are two halves to the GTPase cycle: γ-phosphate hydrolysis and GDP → GTP nucleotide exchange. Both of these steps can be regulated either by inhibition or acceleration of these partial reactions. For example, the Ras protein has an remarkably sluggish intrinsic GTPase activity (Trahey et al. 1987), which can be accelerated at least 10^4- to 10^5-fold by a GTPase activating protein (GAP) (Trahey and McCormick 1987). In addition, there are other regulators of G protein signaling (RGS) that singularly accelerate γ-phosphate hydrolysis, and GDP/GTP exchange stimulators (GESs) and guanine dissociation inhibitors (GDIs) that singularly affect nucleotide exchange (Quilliam et al. 1995; Dohlman and Thorner 1997; Tocque et al. 1997). It is interesting to note that highly conserved amino acid domains have been identified within families of these G protein regulators and their ubiquity suggests that similar factors may exist for the A proteins of which the hMSH2–hMSH6 mismatch repair switch would be a prototypical member. The most likely candidate for an adenine nucleotide regulator of the MutS homologs would be the MutL homologs, which would additionally account for the high conservation of MutL domains. Tests of this hypothesis are currently underway.

Biological switches and the second law of thermodynamics

One could argue that the concept of a singular ON or OFF state in a molecular switch might violate the Second Law of Thermodynamics. The Second Law requires that biochemical systems transit one state to the other by a series of microscopically reversible steps. This idea is based in statistical mechanics as it is applied to a system at equilibrium, which must be applied a priori to enzyme catalyzed biological processes. It is easy to visualize the origins of the principle of microscopic reversibility by considering the consequences were it NOT true. For example, if the rate of A → B were greater than B → A at equilibrium, each of the rates B → C, C → D, and D → A would also have to be greater than their reverse rates to prevent buildup of the concentration of any species, which is not permitted at equilibrium. In this case there would be a preferred direction-of-operation of the reaction cycle. Such a spontaneous cycle in a system at equilibrium (i.e., an engine that spontaneously produces work) is not consistent with the drive toward maximum entropy contained in the Second Law.

There is no violation of the Second Law if the transit from an OFF to ON state (or vice versa) occurs reversibly. The molecular basis for this type of microscopic reversibility can be visualized for the hMSH2–hMSH6 and G-protein switches as reversible nucleotide binding as well as intermediate protein conformational changes that occur while transiting the extreme states. It is these conformational transitions that determine interaction with effectors which is ultimately paid for by the hydrolysis of NTP. More significantly, one can affect the equilibrium of each state experimentally by altering the ratio of NDP/NTP in the absence of any hydrolysis (see Fig. 6 in Gradia et al. 1997). It is also important to note that microscopic reversibility has been directly demonstrated for the gated maxi K^+ ion pump, a molecular switch controlled by similar conformational transitions (Song and Magleby 1994). Thus, molecular switches are both reversible and, at equilibrium, clearly preserving a fundamental tenant of thermodynamics.

The marriage of signal transduction and DNA metabolism

The use of controlled molecular switches appears to pervade all aspects of biology. From the standpoint of DNA metabolism, switch controlled processes appear mechanistically sensible. It is well known that the machines which perform replication, recombination, repair and chromosome segregation are very large and composed of multiple subunits (Alberts 1998). Just like the assembly line for an automobile or an airplane, the assembly of DNA metabolic machines must be done precisely and in a specific order to ensure appropriate function. A series of well defined switches could logically control the progression of such an ordered assembly process. Thus, the same type of switch-controlled cascade events that transduce cellular signals may also control DNA metabolic events. The only apparent difference between these switches is the nucleotide that induces the conformational transitions associated with signaling. At the moment the general rule seems to be guanine nucleotides for cellular signaling events and adenine nucleotides for DNA metabolic signaling events. This dogma raises a number of questions: Why purines? Why adenine versus guanine? Is there really generality to these observations? Do these switches signal more than just the assembly of DNA metabolic machines (i.e., are they signals of damage recognition, etc.)? Is there a connection between these DNA metabolic nucleotide-induced molecular switches and cell-cycle checkpoints? Clearly, communication between the fields of DNA repair and signal transduction will provide the breeding ground for appropriate experiments.

References

Acharya, S., T. Wilson, S. Gradia, M.F. Kane, S. Guerrette, G.T. Marsischky, R. Kolodner, and R. Fishel. 1996. hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. *Proc. Natl. Acad. Sci.* 93,: 13629–13634.

Alani, E., T. Sokolsky, B. Studamire, J.J. Miret, and R.S. Lahue. 1997. Genetic and biochemical analysis of Msh2p–Msh6p: Role of ATP hydrolysis and Msh2p–Msh6p subunit interactions in mismatch base pair recognition. *Mol. Cell. Biol.* 17: 2436–2447.

Alberts, B. 1998. The cell as a collection of protein machines—preparing the next generation of molecular biologists. *Cell* 92: 291–294.

Allen, D.J., A. Makhov, M. Grilley, J. Taylor, R. Thresher, P. Modrich, and J.D. Griffith. 1997. MutS mediates heteroduplex loop formation by a translocation mechanism. *EMBO J.* 16: 4467–4476.

Bokoch, G.M. and C.J. Der. 1993. Emerging concepts in the Ras superfamily of GTP-binding proteins. *FASEB J.* 7: 750–759.

Bronner, C.E., S.M. Baker, P.T. Morrison, G. Warren, L.G. Smith, M.K. Lescoe, M. Kane, C. Earabino, J. Lipford, A. Lindblom et al. 1994. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary nonpolyposis colon cancer. *Nature* 368: 258–261.

Burns, N., B. Grimwade, P.B. Ross-Macdonald, E. Choi, K. Finberg, G.S. Roeder, and M.S. Snyder. 1994. Large-scale analysis of gene expression, protein localization and gene disruption in *Saccharomyces cerevisiae*. *Genes & Dev.* 8: 1087–1105.

Campbell, J.L. and N. Kleckner. 1990. E. coli oriC and the dnaA gene promoter are sequestered from dam methyltransferase following the passage of the chromosomal replication fork. *Cell* 62: 967–979.

Chi, N.W. and R.D. Kolodner. 1994. Purification and characterization of MSH1, a yeast mitochondrial protein that binds to DNA mismatches. *J. Biol. Chem.* 269: 29984–29992.

Cooper, D.L., R.S. Lahue, and P. Modrich. 1993. Methyl-directed mismatch repair is bidirectional. *J. Biol. Chem.* 268: 11823–11829.

Demerec, M., E.L. Lahr, T. Miyake, E. Galehran, E. Balbinder, S. Baric, K. Hashimoto, E.V. Glanville, and J.D. Gross. 1957. Bacterial genetics. *Carnegie Inst. Wash. Yearbook* 370: 390–406.

Dohlman, H.G. and J. Thorner. 1997. RGS proteins and signaling by heterotrimeric G proteins. *J. Biol. Chem.* 272: 3871–3874.

Drummond, J.T., G.-M. Li, M.J. Longley, and P. Modrich. 1995. Isolation of an hMSH2-p160 heterodimer that restores DNA mismatch repair to tumor cells. *Science* 268: 1909–1912.

Fishel, R., A. Ewel, S. Lee, M.K. Lescoe, and J. Griffith. 1994. Binding of mismatched microsatellite DNA sequences by the human MSH2 protein. *Science* 266: 1403–1405.

Fishel, R., M.K. Lescoe, M.R. Rao, N.G. Copeland, N.A. Jenkins, J. Garber, M. Kane, and R. Kolodner. 1993. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* 75: 1027–1038.

Fishel, R. and T. Wilson. 1997. MutS homologs in mammalian cells. *Curr. Opin. Genet. Dev.* 7: 105–113.

Fishel, R.A. and R. Kolodner. 1983. Gene conversion in *Escherichia coli*: The identification of two repair pathways for mismatched nucleotides. *UCLA Symp. Mol. Cell. Biol. New Ser.* 11: 309–324.

Fishel, R.A., E.C. Siegel, and R. Kolodner. 1986. Gene conversion in *Escherichia coli*. Resolution of heteroallelic mismatched nucleotides by co-repair. *J. Mol. Biol.* 188: 147–157.

Friedberg, E.C. 1990. *DNA repair* W.H. Freeman, New York, NY.

Fujii, H. and T. Shimada. 1989. Isolation and characterization of cDNA clones derived from the divergently transcribed gene in the region upstream from the human dihydrofolate reductase gene. *J. Biol. Chem.* 264: 10057–10064.

Gradia, S., S. Acharya, and R. Fishel. 1997. The human mismatch recognition complex hMSH2–hMSH6 functions as a novel molecular switch. *Cell* 91: 995–1005.

Grilley, M., K.M. Welsh, S.S. Su, and P. Modrich. 1989. Isolation and characterization of the *Escherichia coli mutL* gene product. *J. Biol. Chem.* 264: 1000–1004.

Haber, L.T. and G.C. Walker. 1991. Altering the conserved nucleotide binding motif in the *Salmonella typhimurium* MutS mismatch repair protein affects both its ATPase and mismatch binding activities. *EMBO J.* 10: 2707–2715.

Harris, R.S., K.J. Ross, M.J. Lombardo, and S.M. Rosenberg. 1998. Mismatch repair in *Escherichia coli* cells lacking single-strand exonucleases ExoI, ExoVII, and RecJ. *J. Bacte-*

riol. 180: 989–993.
Hill, R.F. 1970. Location of genes controlling excision repair of UV damage and mutator activity in *Escherichia coli* WP2. *Mutat. Res.* 9: 341–344.
Holliday, R.A. 1964. A mechanism for gene conversion in fungi. *Genet. Res.* 5: 282–304.
Hollingsworth, N.M., L. Ponte, and C. Halsey. 1995. MSH5, a novel MutS homolog, facilitates meiotic reciprocal recombination between homologs in *Saccharomyces cerevisiae* but not mismatch repair. *Genes & Dev.* 9: 1728–1739.
Keller, E.B. and P.C. Zamecnik. 1955. The effect of guanosine diphosphate and triphosphate on the incorporation of labeled amino acids into proteins. *J. Biol. Chem.* 221: 45–59.
Kolodner, R. 1996. Biochemistry and genetics of eukaryotic mismatch repair. *Genes & Dev.* 10: 1433–1442.
Kramer, W., B. Kramer, M.S. Williamson, and S. Fogel. 1989. Cloning and nucleotide sequence of DNA mismatch repair gene *PMS1* from *Saccharomyces cerevisiae:* Homology of PMS1 to procaryotic MutL and HexB. *J. Bacteriol.* 171: 5339–5346.
Lahue, R.S., K.G. Au, and P. Modrich. 1989. DNA mismatch correction in a defined system. *Science* 245: 160–164.
Linton, J.P., J.Y. Yen, E. Selby, Z. Chen, J.M. Chinsky, K. Liu, R.E. Kellems, and G.F. Crouse. 1989. Dual bidirectional promoters at the mouse dhfr locus: Cloning and characterization of two mRNA classes of the divergently transcribed Rep-1 gene. *Mol. Cell. Biol.* 9: 3058–3072.
Lu, A.L., S. Clark, and P. Modrich. 1983. Methyl-directed repair of DNA base-pair mismatches in vitro. *Proc. Natl. Acad. Sci.* 80: 4639–4643.
Mankovich, J.A., C.A. McIntyre, and G.C. Walker. 1989. Nucleotide sequence of the *Salmonella typhimurium mutL* gene required for mismatch repair: Homology of *MutL* to *HexB* of *Streptococcus pneumoniae* and to *PMS1* of the yeast *Saccharomyces cerevisiae*. *J. Bacteriol.* 171: 5325–5331.
Marsischky, G.T., N. Filosi, M.F. Kane, and R. Kolodner. 1996. Redundancy of *Saccharomyces cerevisiae* MSH3 and MSH6 in MSH2-dependent mismatch repair. *Genes & Dev.* 10: 407–420.
Miyake, T. 1960. Mutator factor in *Salmonella typhimurium*. *Genetics* 45: 755–762.
Modrich, P. 1989. Methyl-directed DNA mismatch correction. *J. Biol. Chem.* 264: 6597–6600.
———. 1997. Strand-specific mismatch repair in mammalian cells. *J. Biol. Chem.* 272: 24727–24730.
Modrich, P. and R. Lahue. 1996. Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu. Rev. Biochem.* 65: 101–133.
Nathans, D., G. Von Ehrenstein, R. Monro, and F. Lipmann. 1962. Protein synthesis from aminoacyl-soluble ribonucleic acid. *Fed. Proc.* 21: 127–133.
New, L., K. Liu, and G.F. Crouse. 1993. The yeast gene *MSH3* defines a new class of eukaryotic MutS homologues. *Mol. & Gen. Genet.* 239: 97–108.
Nicolaides, N.C., N. Papadopoulos, B. Liu, Y.F. Wei, K.C. Carter, S.M. Ruben, C.A. Rosen, W.A. Haseltine, R.D. Fleischmann, C.M. Fraser et al. 1994. Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. *Nature* 371: 75–80.
Palombo, F., P. Gallinari, I. Iaccarino, T. Lettieri, M. Hughes, A. D'Arrigo, O. Truong, J.J. Hsuan, and J. Jiricny. 1995. GTBP, a 160-kilodalton protein essential for mismatch binding activity in human cells. *Science* 268: 1912–1914.
Prolla, T.A., D.-M. Christie, and R.M. Liskay. 1994. A requirement in yeast DNA mismatch repair for *MLH1* and *PMS1*, two homologs of the bacterial *mutL* gene. *Mol. Cell. Biol.* 14: 407–415.
Quilliam, L.A., R. Khosravi Far, S.Y. Huff, and C.J. Der. 1995. Guanine nucleotide exchange factors: Activators of the Ras superfamily of proteins. *BioEssays* 17: 395–404.
Reenan, R.A.G. and R.D. Kolodner. 1992. Isolation and characterization of two *Saccharomyces cerevisiae* genes encoding homologs of the bacterial HexA and MutS mismatch repair proteins. *Genetics* 132: 963–973.
Siegel, E.C. and V. Bryson. 1967. Mutator gene of *Escherichia coli Brit. J. Bacteriol.* 94: 38–47.
Song, L. and K.L. Magleby. 1994. Testing for microscopic reversibility in the gating of maxi K+ channels using two-dimensional dwell-time distributions [published erratum appears in *Biophys. J.* 1994 Oct; 67[4]:1792]. *Biophy. J.* 67: 91–104.
Sprang, S.R. 1997. G protein mechanisms: Insights from structural analysis. *Annu. Rev. Biochem.* 66: 639–678.
Su, S.-S. and P. Modrich. 1986. *Escherichia coli mutS*-encoded protein binds to mismatched DNA base pairs. *Proc. Natl. Acad. Sci.* 83: 5057–5061.
Tocque, B., I. Delumeau, F. Parker, F. Maurier, M.C. Multon, and F. Schweighoffer. 1997. Ras-GTPase activating protein (GAP): A putative effector for Ras. *Cell Signal* 9: 153–158.
Trahey, M. and F. McCormick. 1987. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science* 238: 542–545.
Trahey, M., R.J. Milley, G.E. Cole, M. Innis, H. Paterson, C.J. Marshall, A. Hall, and F. McCormick. 1987. Biochemical and biological properties of the human N-ras p21 protein. *Mol. Cell. Biol.* 7: 541–544.
Walker, J.E., M. Saraste, M.J. Runswick, and G. NJ. 1982. Distantly related sequences in the alpha-and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* 1: 945–951.
Welsh, K.M., A.L. Lu, S. Clark, and P. Modrich. 1987. Isolation and characterization of the *Escherichia coli mutH* gene product. *J. Biol. Chem.* 262: 15624–15629.
Weinberg, R.A. 1989. Oncogenes, antioncogenes, and the molecular basis of multistep carcinogenesis. *Cancer Res.* 49: 3713–3721.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

What is claimed is:

1. A method of binding a MutS homolog to a mismatched region of a duplex DNA molecule, the method comprising contacting said homolog with said duplex DNA molecule in the presence of a solution comprising an ADP molecule, whereby said homolog binds to said mismatched region.

2. The method of claim 1, wherein said homolog is selected from the group consisting of the *E. coli* MutS protein, an hMSH2 protein dimer, a complex comprising an hMSH2 protein molecule and either an hMSH3 protein molecule or an hMSH6 protein molecule, a yeast MSH2 protein dimer, a complex comprising a yeast MSH2 protein molecule and either a yeast MSH3 protein molecule or a yeast MSH6 protein molecule, a dimer of a rat homolog of hMSH2, a dimer of a *Xenopus* homolog of hMSH2, a dimer of a *Drosophila* homolog of hMSH2, a dimer of a murine homolog of hMSH2, and a complex comprising a murine homolog of hMSH2 and either a murine homolog of hMSH3 or a murine homolog of hMSH6.

3. The method of claim 2, wherein said homolog comprises a complex comprising an hMSH2 protein molecule and an hMSH6 protein molecule.

4. The method of claim 1, wherein said solution comprises at least about 20 micromolar ADP.

5. The method of claim 1, wherein said solution comprises less than about 5 micromolar ATP.

6. The method of claim 1, wherein said solution further comprises ATP and wherein the ratio of the concentration of ADP in said solution to the ratio of the concentration of ATP in said solution is greater than about two.

7. The method of claim 6, wherein said solution is substantially free of ATP.

8. The method of claim 1, wherein said duplex DNA molecule is suspended in said solution.

9. The method of claim 1, wherein said homolog is suspended in said solution.

10. The method of claim 9, wherein said duplex DNA molecule is suspended in said solution.

11. A method of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules, the method comprising

providing a MutS homolog and a solution comprising an ADP molecule and said population of DNA molecules, whereby said homolog binds to said mismatched region, and

segregating said homolog from said population, whereby said duplex DNA molecule is segregated from said population.

12. The method of claim 11, wherein said homolog is bound to a solid support and wherein segregating said homolog from said population comprises rinsing said solid support with a solution which does not comprise said population.

13. The method of claim 11, wherein said population comprises a plurality of cDNA molecules, wherein each of said cDNA molecules is made by reverse transcription of an RNA molecule obtained from an organism.

14. The method of claim 13, wherein said organism is a mammal.

15. The method of claim 14, wherein said mammal is a human.

16. The method of claim 11, further comprising contacting said homolog with a solution comprising an ATP molecule after segregating said homolog from said population, whereby said mismatched region dissociates from said homolog.

17. A method of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence, the method comprising

annealing a first single-stranded DNA molecule and a second single-stranded DNA molecule to form a duplex DNA molecule, wherein said first molecule has said sample sequence and wherein said second molecule has a nucleotide sequence which is complementary to said reference sequence, whereby if there is a difference between said sample sequence and said reference sequence then said duplex DNA molecule has a mismatched region,

thereafter contacting said duplex DNA molecule and a MutS homolog in the presence of a solution comprising an ADP molecule, whereby said homolog binds specifically to said duplex DNA molecule if said duplex DNA molecule comprises said mismatched region, and

thereafter determining whether said homolog binds specifically to said duplex DNA molecule, whereby specific binding of said homolog to said duplex DNA molecule is an indication that there is a difference between said sample nucleotide sequence and said reference nucleotide sequence.

18. The method of claim 17, wherein said sample nucleotide sequence comprises the sequence of a region of a gene obtained from a first organism and wherein said reference nucleotide sequence comprises the sequence of said region obtained from a second organism.

19. The method of claim 18, wherein said first organism and said second organism are the same species.

20. The method of claim 19, wherein each of said first organism and said second organism is a human.

21. The method of claim 17, wherein said sample nucleotide sequence comprises the sequence of a region of a gene obtained from an organism and wherein said reference nucleotide sequence comprises a consensus nucleotide sequence of said region.

22. The method of claim 21, wherein said gene is the human *msh2* gene.

23. A kit for separating a mismatched duplex DNA molecule from a population of molecules, the kit comprising

an MutS homolog;

a linker for binding said homolog to a solid support; and

an ADP molecule.

24. A method of detecting a predisposition of a mammal to carcinogenesis, the method comprising

annealing a first single-stranded DNA molecule and a second single-stranded DNA molecule to form a duplex DNA molecule, wherein said first molecule has the nucleotide sequence of a region of a mammalian gene associated with carcinogenesis and wherein said second molecule has a nucleotide sequence which is complementary to the consensus nucleotide sequence of said region, whereby if there is a sequence difference between said first molecule and said second molecule then said duplex DNA molecule has at least one mismatched region,

thereafter contacting said duplex DNA molecule and a MutS homolog in the presence of a solution comprising an ADP molecule, whereby said homolog binds to said duplex DNA molecule if said duplex DNA molecule comprises a mismatched region, and

thereafter determining whether said homolog binds to said duplex DNA molecule, whereby binding of said homolog to said duplex DNA molecule is an indication of said predisposition of said mammal to carcinogenesis.

25. The method of claim 24, wherein the mammalian gene associated with carcinogenesis is selected from the group consisting of the *msh2* gene, the *msh3* gene, the *msh6* gene, the *mlh1* gene, the *pms2* gene, the *brca1* gene, the *brca2* gene, the *pten* gene, and the *p53* gene.

26. The method of claim 24, wherein said mammalian gene is the human *msh2* gene and wherein said predisposition of a mammal for carcinogenesis is predisposition of a human for hereditary non-polyposis colorectal cancer.

27. A method of fractionating a population of duplex DNA molecules comprising

contacting said population with a MutS homolog in the presence of ADP, whereby said homolog is capable of binding to a duplex DNA molecule of said population if said duplex DNA molecule comprises a mismatched region,

segregating said homolog from said population, and

contacting said homolog with a dissociation solution comprising an ATP molecule, whereby said duplex DNA molecule dissociates from said homolog, whereby said population is fractionated.

28. A method of selectively amplifying at least one mismatched duplex DNA molecule of a population of duplex DNA molecules, the method comprising

contacting said population with a MutS homolog in the presence of a solution comprising an ADP molecule, whereby said homolog binds to said at least one mismatched duplex DNA molecule,

thereafter segregating said homolog from said population, whereby each said at least one mismatched duplex DNA molecule is segregated from said population, and

thereafter amplifying each said at least one mismatched duplex DNA molecule using a PCR technique, whereby each of said at least one mismatched duplex DNA molecule is selectively amplified.

29. A method of detecting the presence of a genetic polymorphism in the genome of an animal comprising

amplifying a region of each of two copies of a gene of said animal to yield an amplified first copy and an amplified second copy,

thereafter mixing and denaturing each of said first copy and said second copy to yield a first mixture of nucleic acids comprising a first sense copy, a first antisense copy, a second sense copy, and a second antisense copy,

thereafter annealing the nucleic acids in said first mixture to yield a second mixture of nucleic acids comprising said first copy, said second copy, a first sense-second antisense duplex DNA molecule, and a second sense-first antisense duplex DNA molecule, whereby if said first sense copy and said second antisense copy are not completely complementary then said first sense-second antisense duplex DNA molecule will have a first mismatched region, and whereby if said second sense copy and said first antisense copy are not completely complementary then said second sense-first antisense duplex DNA molecule will have a second mismatched region,

thereafter contacting said second mixture with a MutS homolog in the presence of a solution comprising an ADP molecule, whereby said homolog is capable of binding to either of said first mismatched region or said second mismatched region,

thereafter segregating said homolog from said second mixture, and

thereafter detecting the presence or absence of either of said first sense-second antisense duplex DNA molecule or said second sense-first antisense duplex DNA molecule, whereby the presence of either of said first sense-second antisense duplex DNA molecule or said second sense-first antisense duplex DNA molecule is an indication that said animal has a genetic polymorphism.

30. A composition for segregating a mismatched duplex DNA molecule from a population of molecules, said composition comprising a MutS homolog bound to a solid support, wherein said support is in liquid contact with a solution comprising an ADP molecule.

31. A kit for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence, said kit comprising

a pair of primers adjacent said region for amplifying said region;

a duplex DNA molecule having said reference nucleotide sequence;

a solid support;

a MutS homolog;

a linker for binding said MutS homolog to said solid support; and

an ADP molecule.

32. A nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene.

33. The nonhuman mammal of claim 32, wherein said mammal is a rodent.

34. The nonhuman mammal of claim 33, wherein said nonhuman mammal is a mouse.

35. The nonhuman mammal of claim 32, wherein said mammal has an average survival time shorter than the average survival time of a nullizygous *Msh2* mammal of the same species.

36. The nonhuman mammal of claim 32, wherein said mammal has an average survival time shorter than the average survival time of a nullizygous *p53* mammal of the same species.

37. The nonhuman mammal of claim 32, wherein said mammal exhibits female-specific embryonic lethality.

38. A method of making a nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene, said method comprising mating a first mammal of a species, said first mammal comprising at least one null allele of the *Msh2* gene of said species with a second mammal of said species, said second mammal comprising at least one null allele of the *p53* gene of said species.

39. A method of identifying a compound which affects tumorigenesis in a mammal comprising

administering said compound to a first nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene, and

comparing tumor incidence in said first transgenic mammal with tumor incidence in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered said compound, wherein a difference in tumor incidence in said first transgenic mammal compared with tumor incidence in said second transgenic mammal is an indication that said compound affects tumorigenesis in said mammal.

40. A method of identifying a compound which affects apoptosis in a mammal comprising

administering said compound *in utero* to a first nonhuman mammal embryo which is nullizygous for both the *Msh2* gene and the *p53* gene, and

comparing embryonic development in said first transgenic mammal embryo with embryonic development in a second nonhuman mammal which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered said compound, wherein a difference in embryonic development of said first transgenic mammal compared with embryonic development of said second transgenic mammal is an indication that said compound affects apoptosis in said mammal.

41. A method of identifying a compound which affects the aging process in a mammal comprising

administering said compound *in utero* to a first nonhuman mammal embryo which is nullizygous for both the *Msh2* gene and the *p53* gene, and

comparing embryonic development in said first transgenic mammal embryo with embryonic development in a second nonhuman mammal

which is nullizygous for both the *Msh2* gene and the *p53* gene and which is not administered said compound, wherein a difference in embryonic development of said first transgenic mammal compared with embryonic development of said second transgenic mammal is an indication that said compound affects the aging process in said mammal.

42. A cell line which is nullizygous for both the *Msh2* gene and the *p53* gene, wherein said cell line is made by culturing a cell obtained from the nonhuman mammal of claim 32.

43. A method of binding a MutS homolog to a mismatched region of a duplex DNA molecule, the method comprising contacting said homolog with said duplex DNA molecule in the presence of a solution comprising ADP and a MutL homolog derivative, whereby said MutS homolog binds to said mismatched region.

44. A method of segregating a duplex DNA molecule having a mismatched region from a population of DNA molecules, the method comprising

providing a MutS homolog and a solution comprising a MutL homolog derivative, ADP, and said population of DNA molecules, whereby said homolog binds to said mismatched region, and

segregating said homolog from said population, whereby said duplex DNA molecule is segregated from said population.

45. A method of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence, the method comprising

annealing a first single-stranded DNA molecule and a second single-stranded DNA molecule to form a duplex DNA molecule, wherein said first molecule has said sample sequence and wherein said second molecule has a nucleotide sequence which is complementary to said reference sequence,

whereby if there is a difference between said sample sequence and said reference sequence then said duplex DNA molecule has a mismatched region,

thereafter contacting said duplex DNA molecule and a MutS homolog in the presence of a solution comprising a MutL homolog derivative and ADP, whereby said MutS homolog binds specifically to said duplex DNA molecule if said duplex DNA molecule comprises said mismatched region, and

thereafter determining whether said MutS homolog binds specifically to said duplex DNA molecule, whereby specific binding of said MutS homolog to said duplex DNA molecule is an indication that there is a difference between said sample nucleotide sequence and said reference nucleotide sequence.

46. A kit for separating a mismatched duplex DNA molecule from a population of molecules, the kit comprising

a MutS homolog;

a MutL homolog derivative;

a linker for binding said homolog to a solid support; and

an ADP molecule.

47. A method of detecting a predisposition of a mammal to carcinogenesis, the method comprising

annealing a first single-stranded DNA molecule and a second single-stranded DNA molecule to form a duplex DNA molecule, wherein said first molecule has the nucleotide sequence of a region of a mammalian gene associated with carcinogenesis and wherein said second molecule has a nucleotide sequence which is complementary to the consensus nucleotide sequence of said region, whereby if there is a sequence difference between said first molecule and said second molecule then said duplex DNA molecule has at least one mismatched region,

thereafter contacting said duplex DNA molecule and a MutS homolog in the presence of a solution comprising a MutL homolog derivative and ADP,

whereby said MutS homolog binds to said duplex DNA molecule if said duplex DNA molecule comprises a mismatched region, and

thereafter determining whether said MutS homolog binds to said duplex DNA molecule, whereby binding of said MutS homolog to said duplex DNA molecule is an indication of said predisposition of said mammal to carcinogenesis.

48. A method of fractionating a population of duplex DNA molecules comprising

contacting said population with a MutS homolog in the presence of a MutL homolog derivative and ADP, whereby said MutS homolog is capable of binding to a duplex DNA molecule of said population if said duplex DNA molecule comprises a mismatched region,

segregating said MutS homolog from said population, and

contacting said MutS homolog with a dissociation solution comprising an ATP molecule, whereby said duplex DNA molecule dissociates from said homolog, whereby said population is fractionated.

49. A method of selectively amplifying at least one mismatched duplex DNA molecule of a population of duplex DNA molecules, the method comprising

contacting said population with a MutS homolog in the presence of a solution comprising a MutL homolog derivative and ADP, whereby said MutS homolog binds to said at least one mismatched duplex DNA molecule,

thereafter segregating said MutS homolog from said population, whereby each said at least one mismatched duplex DNA molecule is segregated from said population, and

thereafter amplifying each said at least one mismatched duplex DNA molecule using a PCR technique, whereby each of said at least one mismatched duplex DNA molecule is selectively amplified.

50. A method of detecting the presence of a genetic polymorphism in the genome of an animal comprising

amplifying a region of each of two copies of a gene of said animal to yield an amplified first copy and an amplified second copy,

thereafter mixing and denaturing each of said first copy and said second copy to yield a first mixture of nucleic acids comprising a first sense copy, a first antisense copy, a second sense copy, and a second antisense copy,

thereafter annealing the nucleic acids in said first mixture to yield a second mixture of nucleic acids comprising said first copy, said second copy, a first sense-second antisense duplex DNA molecule, and a second sense-first antisense duplex DNA molecule, whereby if said first sense copy and said second antisense copy are not completely complementary then said first sense-second antisense duplex DNA molecule will have a first mismatched region, and whereby if said second sense copy and said first antisense copy are not completely complementary then said second sense-first antisense duplex DNA molecule will have a second mismatched region,

thereafter contacting said second mixture with a MutS homolog in the presence of a solution comprising a MutL homolog derivative and ADP, whereby said MutS homolog is capable of binding to either of said first mismatched region or said second mismatched region,

thereafter segregating said MutS homolog from said second mixture, and

thereafter detecting the presence or absence of either of said first sense-second antisense duplex DNA molecule or said second sense-first antisense duplex DNA molecule, whereby the presence of either of said first sense-second antisense duplex DNA molecule or said second sense-first antisense duplex DNA molecule is an indication that said animal has a genetic polymorphism.

51. A composition for segregating a mismatched duplex DNA molecule from a population of molecules, said composition comprising a MutS homolog bound to a solid support, wherein said support is in liquid contact with a solution comprising a MutL homolog derivative and ADP.

52. A kit for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence, said kit comprising

a pair of primers adjacent said region for amplifying said region;

a duplex DNA molecule having said reference nucleotide sequence;

a solid support;

a MutS homolog;

a MutL homolog derivative;

a linker for binding said MutS homolog to said solid support; and

an ADP molecule.

53. A method of any one of claims 43 to 52, wherein said MutL homolog is selected from the group consisting of yeast MutL homologs MLH1, MLH2, PMS1 and human MutL homologs hMLH1, hPMS1, and hPMS2

ABSTRACT OF THE DISCLOSURE

Compositions, and products comprising a MutS homolog which binds to a mismatched region of a duplex DNA molecule in the presence of ADP are provided, as are methods of binding MutS homologs to mismatched DNA in the presence of ADP. The use of MutL homolog derivatives in combination with MutS homologs is also included. Nonhuman mammals which are nullizygous for both the *Msh2* gene and the *p53* gene are also provided, as are methods of making and using the same.

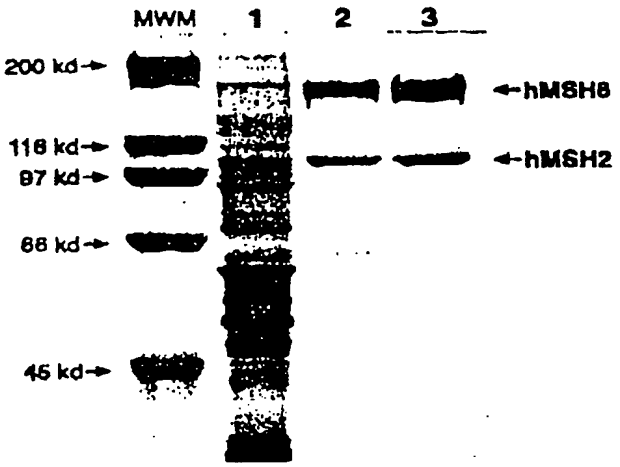


Figure 1

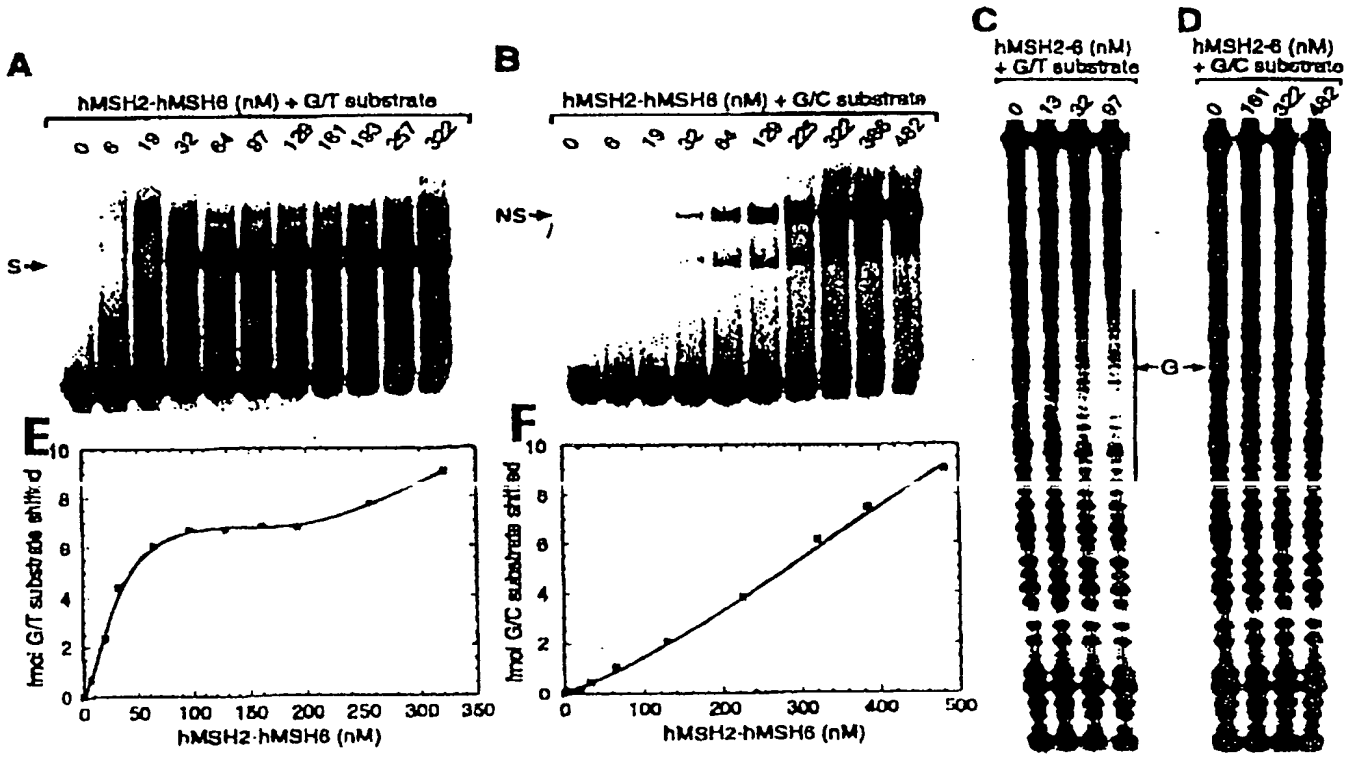

A
hMSH2-hMSH6 (nM) + G/T substrate
0 6 13 32 64 97 129 161 193 257 321
S
B
hMSH2-hMSH6 (nM) + G/C substrate
0 6 13 32 64 129 193 257 386 482
NS
C
hMSH2-6 (nM)
+ G/T substrate
0 13 32 97
G
D
hMSH2-6 (nM)
+ G/C substrate
0 161 321 482
E
fmol G/T substrate shifted
hMSH2-hMSH6 (nM)
F
fmol G/C substrate shifted
hMSH2-hMSH6 (nM)

Figure 2

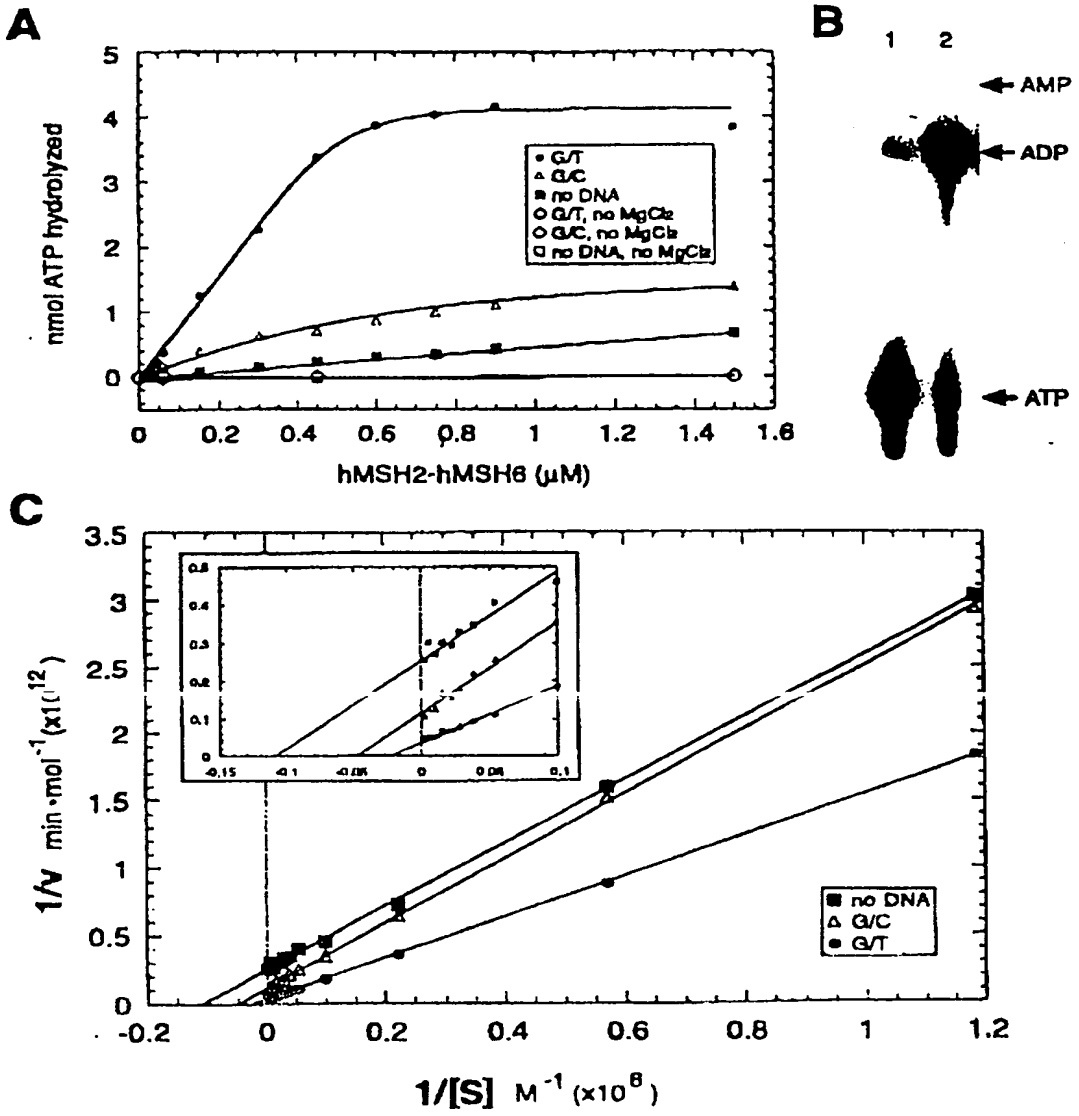

A
nmol ATP hydrolyzed
hMSH2-hMSH6 (μM)
G/T
G/C
no DNA
G/T, no MgCl2
G/C, no MgCl2
no DNA, no MgCl2
B
1
2
AMP
ADP
ATP
C
1/v min·mol-1 (x10-12)
1/[S] M-1 (x10-6)
no DNA
G/C
G/T

Figure 3

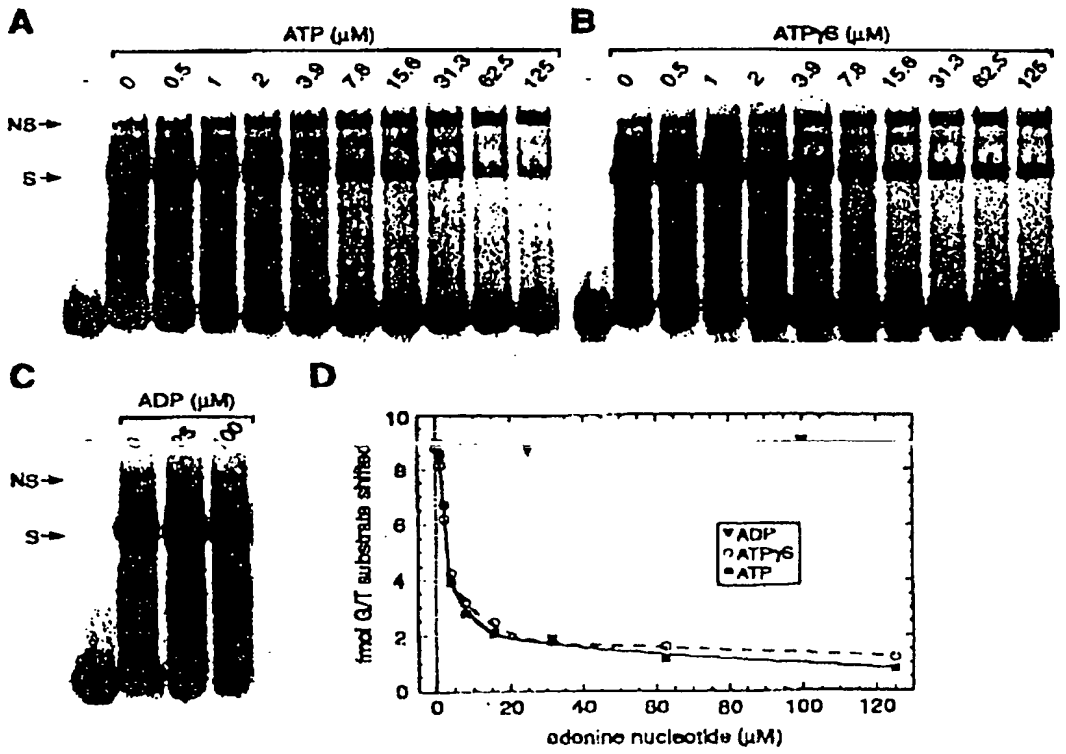

A
ATP (μM)
0 0.5 1 2 3.9 7.8 15.6 31.3 62.5 125
NS
S
B
ATPγS (μM)
0 0.5 1 2 3.9 7.8 15.6 31.3 62.5 125
C
ADP (μM)
NS
S
D
fmol Q/T substrate shifted
adonine nucleotide (μM)
ADP
ATPγS
ATP

Figure 4

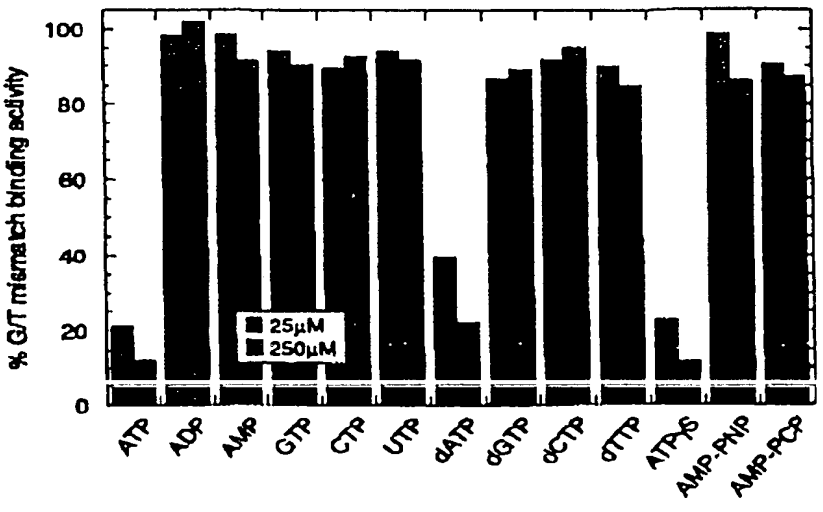

% G/T mismatch binding activity
100
80
60
40
20
0
25μM
250μM
ATP
ADP
AMP
GTP
CTP
UTP
dATP
dGTP
dCTP
dTTP
ATPγS
AMP-PNP
AMP-PCP

Figure 5

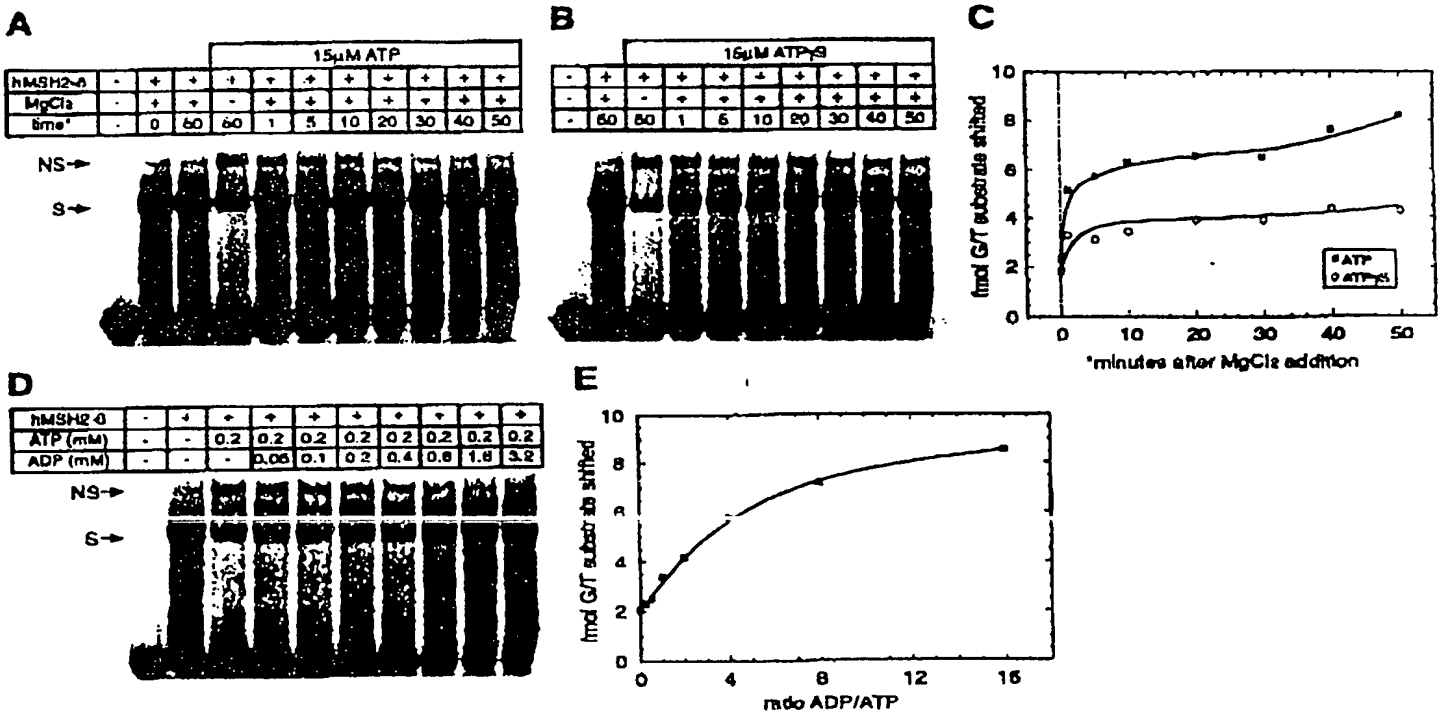


Figure 6

Figure 7

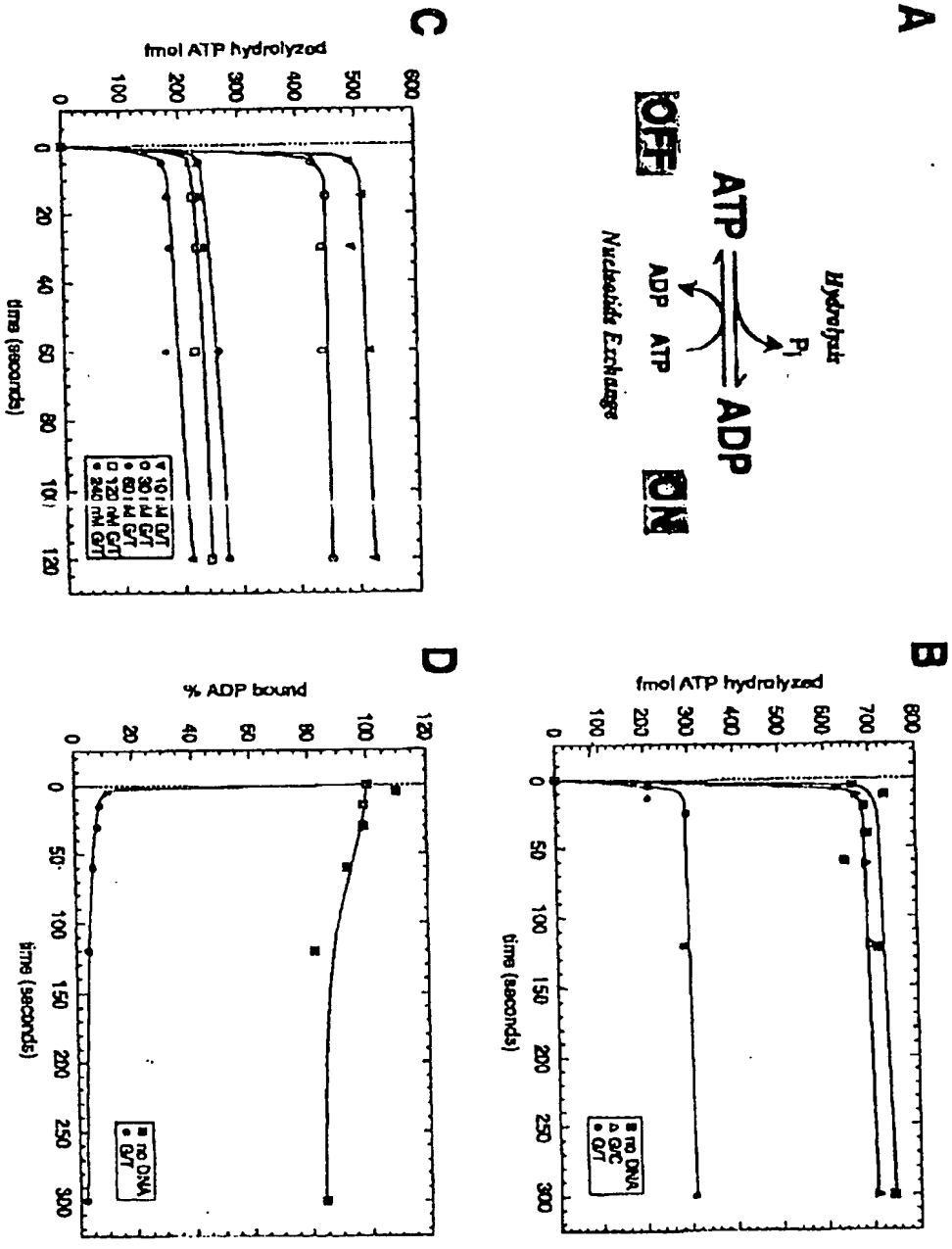
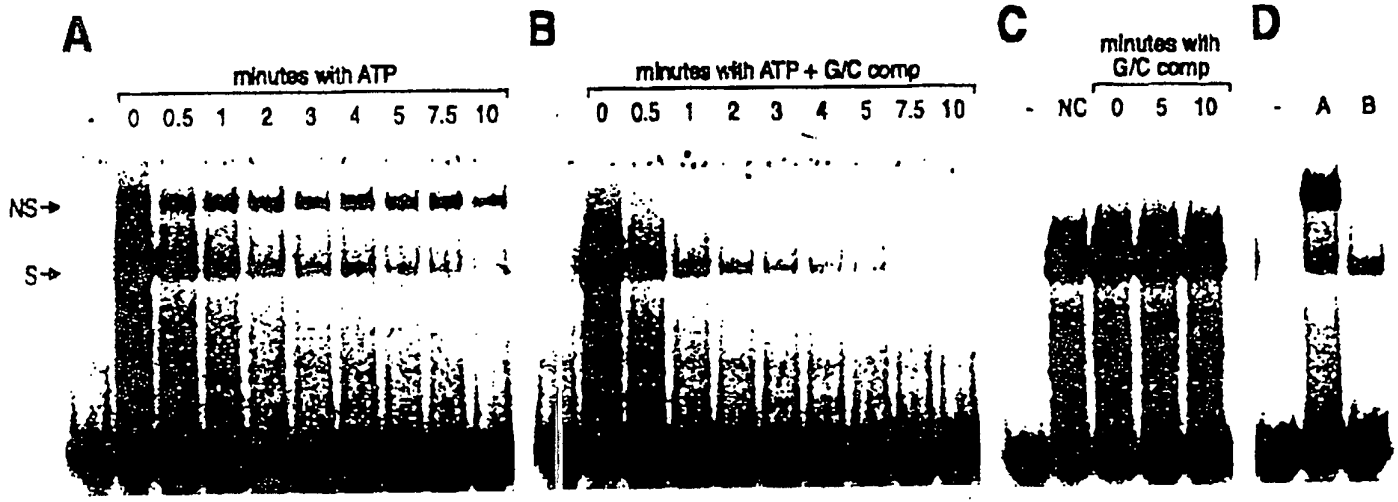

A
ATP
ADP
Pi
Hydrolysis
Nucleotide Exchange
OFF
ON
ADP ATP
B
fmol ATP hydrolyzed
time (seconds)
no DNA
GJC
GJT
C
fmol ATP hydrolyzed
time (seconds)
10 nM GJT
30 nM GJT
120 nM GJT
240 nM GJT
D
% ADP bound
time (seconds)
no DNA
GJT

Figure
8


A
minutes with ATP
- 0 0.5 1 2 3 4 5 7.5 10
NS→
S→
B
minutes with ATP + G/C comp
0 0.5 1 2 3 4 5 7.5 10
C
minutes with G/C comp
- NC 0 5 10
D
- A B

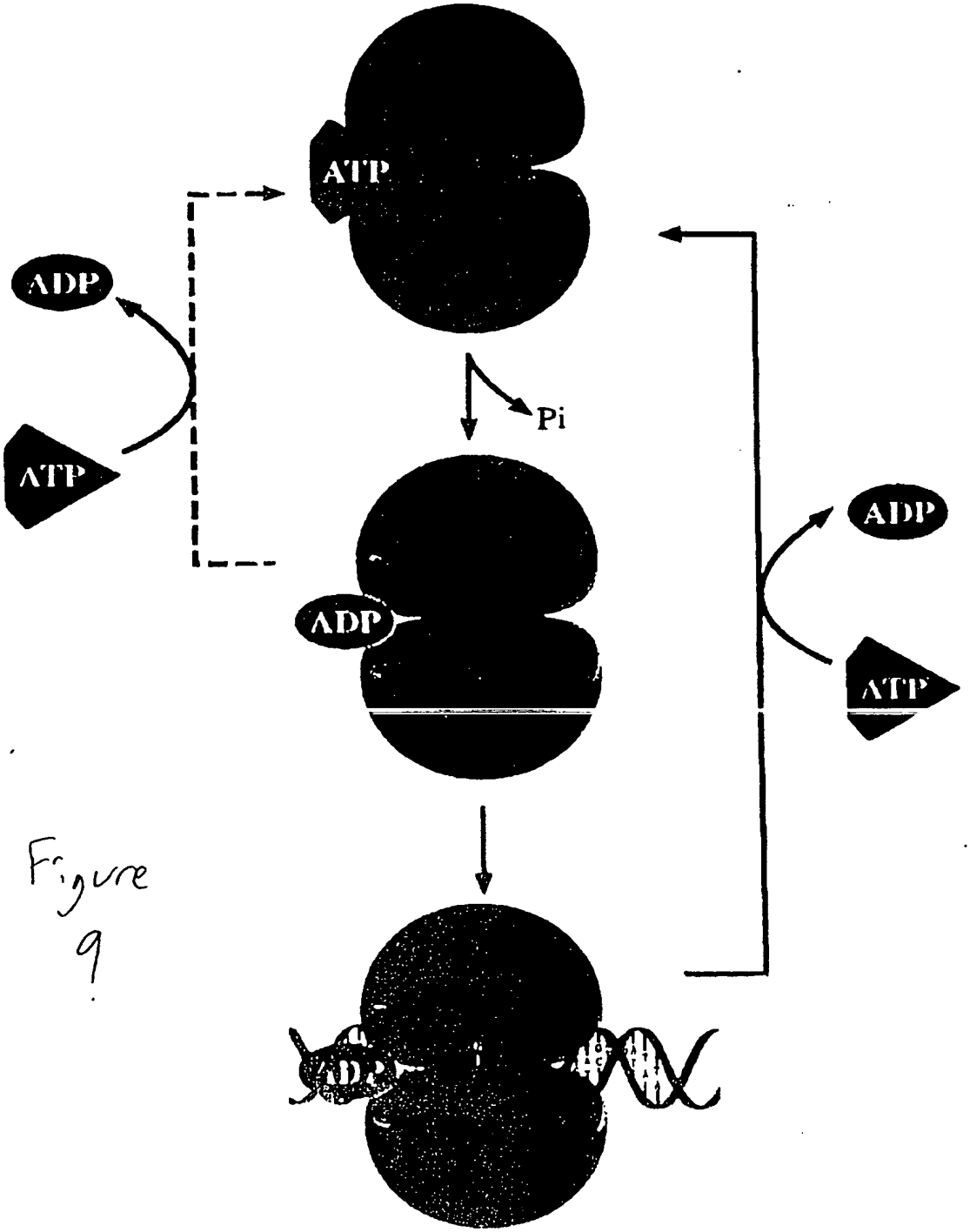


Figure 9

Figure 10

SEQ ID NO:1

5' CGGCGAATTC CACCAAGCTT GATCGCTCGA GGTACCAGG 3'

SEQ ID NO: 2

5' CCTGGTACCT CGAGCGATCA AGCTTGGTGG AATTCGCCG 3'

SEQ ID NO: 3

5' CCTGGTACCT CGAGCGATCG AGCTTGGTGG AATTCGCCG 3'

SEQ ID NO:4

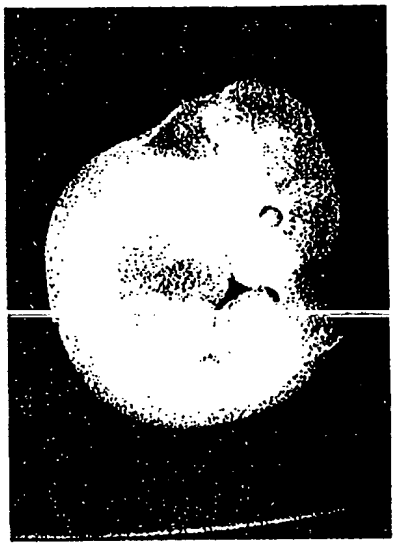
5' AAAGCTGGAG CTGAAGCTTA GCTTAGGATC ATCGAGGATC
GAGCTCGGTG CAATTCAGCG GTACCCAATT CGCCCTATAG T 3'

SEQ ID NO: 5

5' ACTATAGGGC GAATTGGGTA CCGCTGAATT GCACCGAGCT
CGATCCTCGA TGATCCTAAG CTAAGCTTCA GCTCCAGCTT T 3'

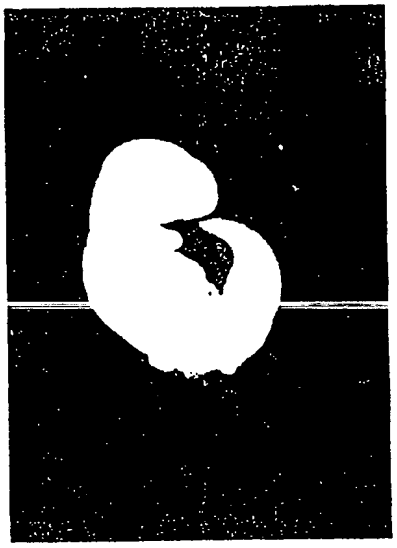
SEQ ID NO: 6

5' ACTATAGGGC GAATTGGGTA CCGCTGAATT GCACCGAGCT
TGATCCTCGA TGATCCTAAG CTAAGCTTCA GCTCCAGCTT T 3'



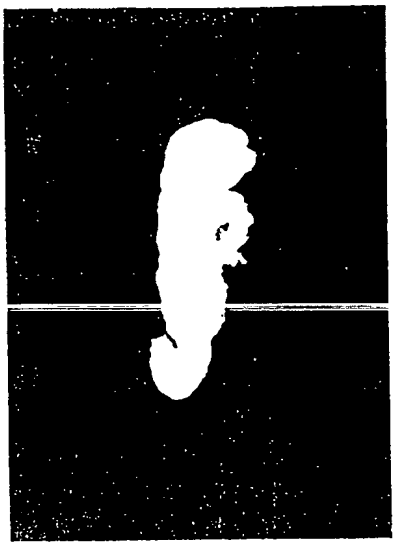
Figure

11 A



Figure

11 B



Figure

11 C

Figure

11 D

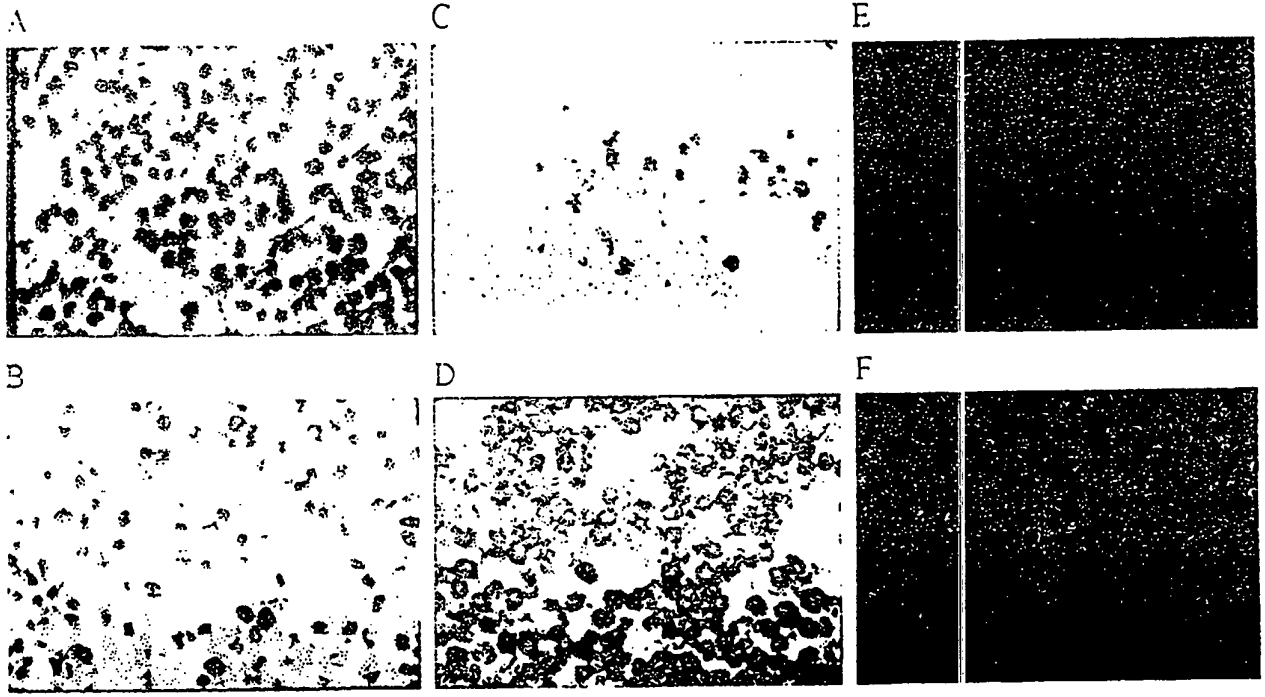


Figure 12

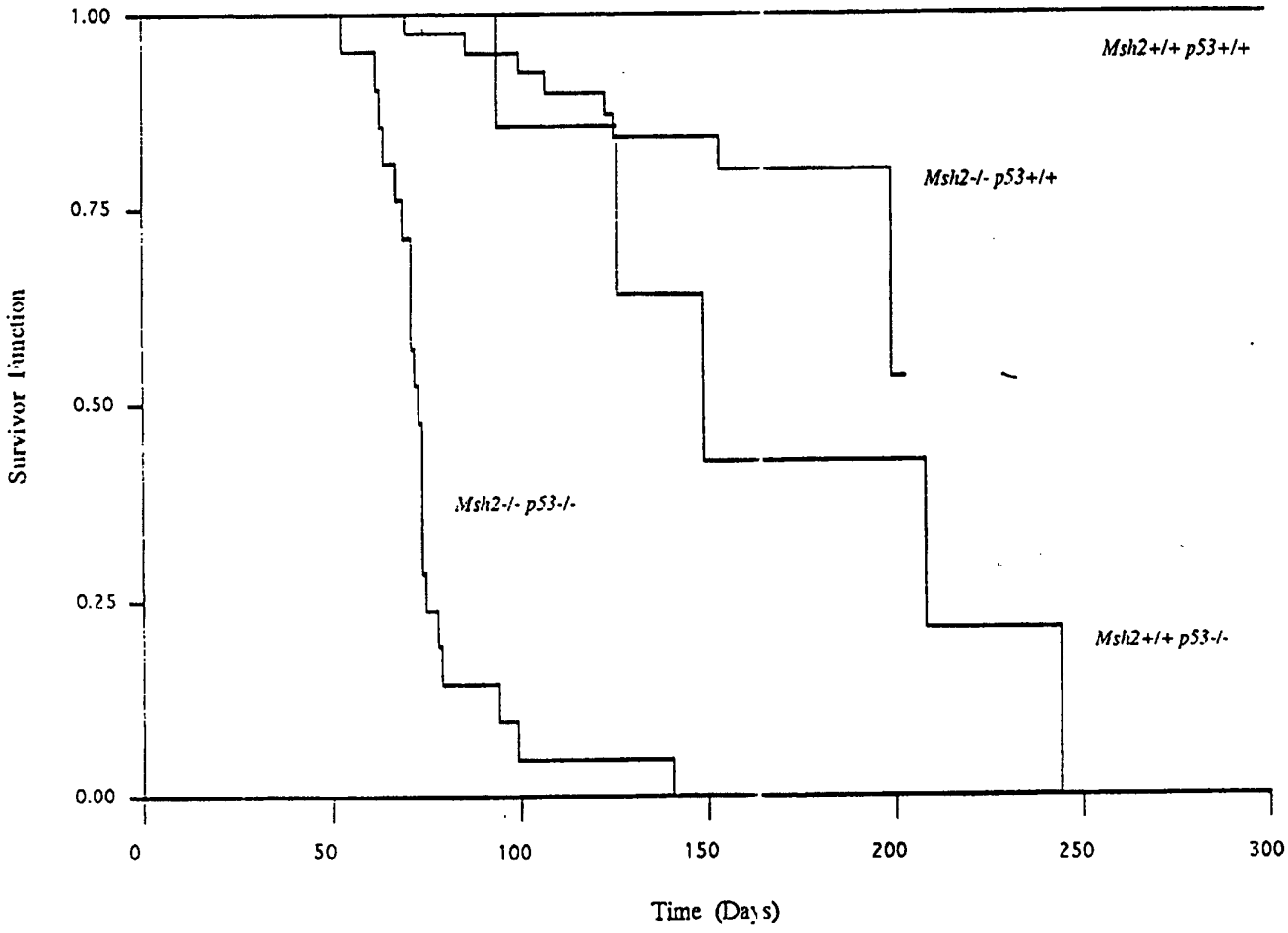

1.00
0.75
0.50
0.25
0.00
Survivor Function
0
50
100
150
200
250
300
Time (Days)
Msh2+/+ p53+/+
Msh2-/- p53+/+
Msh2-/- p53-/-
Msh2+/+ p53-/-

Figure 13

Figure 14

Mammalian MutS Homolog 5 is Required for Chromosome Pairing in Meiosis

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MutS homolog 5 (MSH5) is a member of a family of proteins that are known to be involved in DNA mismatch repair. Unlike some other members of this family, which are ubiquitously expressed, MSH5 expression is restricted with high expression in gonadal tissues. To assess the role of MSH5, we developed mice carrying a null mutation in the MSH5 gene. Mice homozygous for this mutation are viable but are sterile. In males the sterility is the result of the failure of pairing of homologous chromosomes although they do associate with proteins of the synaptonemal complex and Rad51, a marker for early recombination nodules. This results in the complete loss of post-zygotene spermatocytes, no sperm and a 70% reduction of testis size. In females, oocytes are present up to 3 days post-partum but are rapidly depleted with a complete loss by day 6 post-partum. The ovaries are of normal size at birth, but degenerate and are entirely absent in the adult. The time of meiotic blockage in MSH5 mutant mice implicates this protein in a completely novel function of initiation of homologous pairing in meiosis. Our results also show that meiotic synaptic failure in embryonic ovaries leads to a failure in developmental remodeling of the ovary, loss of the oocyte pool and complete ovarian degeneration in early adulthood, providing an explanation of the ovarian phenotype in Tuner syndrome patients.

Mismatched bases in DNA result from a number of mechanisms including errors in DNA replication, genetic recombination and modification of DNA or nucleotides. The enzymatic mechanisms that are capable of repairing mismatched nucleotides in DNA are reletively well conserved in prokaryotes and eukaryotes. The bacterial DNA mismatch repair systems typified by those found in E. coli are among the simplest and best understood. Among the systems that repair mismatches, the MutHLS system has been most extensively studied. This system is capable of repairing both single nucleotide mismatches as well as small insertion/deletion mismatches (for reviews, see Modrich 1991; Kolodner et al., 1995, 1996; Modrich and Lahue, 1996). In this system, the MutS protein binds to DNA at the site of the mismatch and is required for mismatch recognition. A second protein, MutL, interacts with MutS when MutS is bound to a mispaired base and this ternery complex plays a crucial role in the activity of the proteins that are responsible for the excision and resynthesis steps of mismatch repair.

A remarkable observation made in eukaryotes, initially in yeast and later in humans, is that MutS and MutL related proteins that are essential components of the mismatch repair system have been conserved between eukaryotes and prokaryotes. The repair system is, however, more complex and involves several MutS and MutL homologs. In the yeast Saccharomyces cerevisiae there are six homologs of the mispair recognition component of the reaction, MutS (Reenan and Kolodner, 1992a, 1992b; New et al., 1993; Ross-McDonald and Roeder, 1994; Hollingsworth et al., 1995; Marsischky et

al., 1996) designated MutS homolog (MSH) 1-6. There are four known homologs of the MutL gene in yeast, designated MLH1-3 and PMS1 **(for a review see Crouse, 1998)**. The mammalian genome encodes homologs for most of these genes including 5 of the 6 MutS homologs, MSH2-6. The products of MSH2, MSH3, MSH6, as well as MLH1 and PMS1 (PMS2 in humans) are clearly involved in DNA mismatch repair. However, in contrast to bacterial mismatch repair were a single MutS protein is required, eukaryotic mismatch repair utilizes MSH2-MSH3 and MSH2-MSH6 complexs to recognize different types of **mismatches** in DNA and an MLH1-PMS1 complex that interacts with the MSH2 complex instead of a single MutL protein **(Kolodner et al., 1995, 1996; Modrich and Lahue, 1996)**.

In S. cerevisiae, there is considerable evidence that MutS and MutL related proteins are required for normal meiosis. The roles of these proteins in meiosis include their requirement for the repair of mispaired bases in meiotic recombination intermediates as well as apparantly a role in promoting crossing over that is distinct from a role in mismatch repair (**Petes** et al., 1991; Reenan and Kolodner, 1992; Prolla et al., 1994; Ross-McDonald and Roeder, 1994; Hollingsworth et al., 1995; Williamson et al., 1985; **Alani et al, 1994**). One impetus for the study of mismatch repair genes in humans came from the identification of the roles of MSH2 and MLH1 in a cancer predisposition syndrome, hereditary non-polyposis colon cancer (HNPCC) (Lynch syndrome) as well as in the development of some types of sporadic cancers. However, it is also important to understand the roles of these genes in meiosis since many of the

meiotic defects seen in mutant S. cerevisiae strains resemble the types of meiotic defects seen in some types of infertility in humans.

To understand the role of the mismatch repair genes in DNA repair, cancer predisposition and meiosis, we and several other investigators have begun to generate mice by gene targeting that are mutant in each of the genes encoding MutS and MutL homologs and examine their phenotypes. Mice which carry mutations in Msh2 (deWind et al., 1995; Reitmair et al., 1995; Fodde et al., unpublished observations), mlh1 (Edelmann et al., 1996; Baker et al., 1996), PMS2 (Baker et al., 1995), PMS1 (Reference) and Msh6 (Edelmann et al., 1997) have been described. Although all of the genes examined to date encode proteins that function in the same complex involved in recognition of mismatches in DNA, mice lacking different genes have some common and some distinct features. Qualitatively, Msh2 -/-, Mlh1 -/-, Msh6 -/- and PMS2 -/- show a disposition to develop tumors, although the degree of this predisposition and the time after birth when tumors develop differ. Mice lacking PMS1 appear to be normal in this regards.

A remarkable observation made during the studies of the mutant mice is that some of them have meiotic defects while others do not. Mice with mutations in the MSH gene family (MSH2 and MSH6), in the homozygous state, are viable and fully fertile (deWind et al., 1995; Reitmair et al., 1995; Edelmann et al., 1997). Mice with a homozygous mutation in PMS2 show abnormal chromosome pairing during male meiosis and are male sterile whereas the females are fertile (Baker et al., 1995). Mice with mutations in the Mlh1 gene are viable but both sexes are sterile. Normal

chromosome pairing was observed in pachytene but most of the cells fail to progress beyond pachytene and seem to be shunted into an apoptotic pathway. A few cells that survive this shunt show premature separation of homologous chromosomes.

How could inactivation of genes (MSH2, MSH6 and MLH1) whose products are known to interact yield related repair defects and cancer predisposition phenotypes, cause extremely diverse meiotic phenotypes? A possible explanation is that MLH1, MSH2 and MSH6 interact and are members of a complex involved in repair of DNA mismatches in somatic cells but that MLH1 employs a different member of the MSH family as its partner in meiosis. The fact that in yeast mutations in MSH4 and MSH5 cause a meiotic phenotype (Ross-McDonald and Roeder, 1994; Hollingsworth et al, 1995) and that these genes are expressed in germ cells in mammals (Paquis et al., 1997; Winand et al., 1998) suggested that one or both of these gene products may be the partners for Mlh1.

To ascertain the role of MSH4 and MSH5 in mammals, we initiated an effort to generate mice with mutations in each of these genes. We now report the generation and characterization of mice with a null mutation in Msh5. Msh5 -/- mice are viable but males and females are sterile. Meiosis in these mice is severely affected where the chromosomes fail to pair. This meiotic failure leads to a rapid and progressive attrition of testes and ovaries. Our results show that normal MSH5 function is essential for meiotic progression and gonadal maintenance.

RESULTS

Isolation of mouse cDNA and genomic clones for MSH5. The isolation of human **MSH5 cDNA, its genomic** organization and expression patterns were described (**Winand** et al., 1998; Doggett et al., 1998). **As part of the present study, 2.7 kb of mouse MSH5 cDNA sequence was defined by sequencing 5′- and 3′-RACE products** (Figure 1). **This mouse cDNA contains an open reading frame corresponding to 834 a.a. The coding region of the mouse cDNA shares 86% sequence identity and the predicted amino acid sequence shares 89% identity with the human cDNA and predicted amino acid sequence, respectively.** The high level of conservation in the DNA sequence and the deduced amino acid sequence suggest functional conservation in the two species. **In addition, we determined the sequence of introns 10, 17, 18, 19 and 21 by sequencing selected regions of P1 clones containing the mouse MSH5 gene and found that the mouse and human genes ahd the same structural organization in these regions (data not shown).**

Generation of mice with a disrupted Msh5 gene. A 344 bp PstI fragment of the mouse cDNA which contains exon 16 of the gene was used to screen a mouse genomic λ phage library derived from the strain 129/Ola. From one of the positive clones, we isolated a 3.8 Kb HindIII fragment that contained exons 15 and 16 of the Msh5 gene. The gene targeting scheme that we used to generate mouse embryonic stem (ES) cells is shown in Figure 2. **The** gene targeting vector designated pMSsh5ex16 was constructed by insertion of a PGK-hygromycin gene cassette into the unique Aat II site in exon 16 of the gene. The hygromycin expression cassette is the opposite transcriptional orientation to

that of Msh5. As a result, any transcript that is derived from the modified gene would contain a number of in-frame stop codons. pMsh5ex16 was linearized and introduced into ww6 ES cells. One hundred and forty-four of the hygromycin resistant clones were screened by PCR for the desired gene modification event. Six of the 144 (4%) had the desired modification. Two of these cell lines, MSH5-1 and MSH5-52 were injected into blastocysts derived from C57BL/6 females. Both cell lines yielded robust chimeras which transmitted the modified locus through their germ line.

Homozygous Msh5 -/- mice are viable. To examine the effect of the Msh5 gene mutation on the viability of mice, we interbred Msh5 +/- mice. The offspring from several such matings were genotyped by PCR and in some cases by Southern blotting. For Southern blotting, tail DNA was digested with NsiI and hybridized with a probe located outside the targeted region. Representatative results are shown in Figure 2. The wild-type locus is represented by a 2.0 Kb fragment while the modified locus is represented by a 4.0 Kb band. We obtained three classes of mice corresponding to +/+, +/- and -/- genotypes. Of 606 mice that were examined, 184 were +/+, 275 were +/- and 147 were -/-. These results show that mice which have a disrupted Msh5 gene in the homozygous state are viable.

Msh5 -/- mice do not express Msh5 transcripts or protein. To examine the expression of the modified Msh5 locus, we prepared RNA from testes of 24 day old Msh 5 +/+ and -/- mice and did Northern blot analysis with different probes. The results are shown in Figure 3. When an Msh5 probe corresponding to exons 3-8 of the gene was used, RNA from +/+ mice gave a 2.0 Kb band. No such band was detected in RNA

from testes of -/- mice. To ensure that testes specific RNA was indeed used, we hybridized the same blots with a probe corresponding to Msh4 and §-actin. Testes RNA from both +/- and -/- mice contained equivalent amounts of §-actin. Both samples also were positive for Msh4 transcripts, although their levels are lower in the mutant homozygotes. We believe that the reduced Msh4 transcripts reflect a reduction in germ cells in 24 days old males (see below).

We also examined for the presence of MSH5 protein in testes. Protein extracts from testes of 24 days old males were tested by Western blot analysis using a COOH and specific antibody (Figure 3). The antibody reacted with the expected 93 Kd MSH5 protein in extracts from +/+ and +/- mice but no such band was detectable in extracts from -/- mice. Taken together, the Northern and Western blot data suggest that the modified Msh5 locus does not produce a functional MSH5 protein.

Msh5 -/- males are sterile. The restricted localization of *msh5* to the gonads of mice and humans (Fig. X; Winand et al, 1998), together with the meiotic phenotype of *msh5* yeast mutants, suggests a role for *msh5* in mammalian meiosis. Detailed analysis of msh5 mRNA expression in prepubertal testis is consistent with this role. The onset of *msh5* expression is co-incident with the onset of the first wave of meiosis in the mouse testis, beginning at day 11 postpartum (pp) and peaking at day 23-26 pp (Fig. 4A). This temporal expression pattern closely mirrors the acquisition of early spermatocytes in the seminiferous tubules and peaks at a time when the proportion of cells in prophase I is maximal, suggesting that any role of msh5 in meiosis must be limited to early meiosis. Therefore, we analysed spermatogenesis and male sexual behaviour in msh5-

deficient mice. Homozygous mutant males demonstrated normal sexual behavior in the presence of wildtype females, and their mating frequency, as assessed by the formation of vaginal plugs in receptive females, was identical to that of +/+ and +/- males. However, such matings never led to pregnancies. Examintation of the testes of these *msh5* -/- males showed that they were 70% smaller than age-matched controls and, in the epididymides there was a complete lack of spermatozoa while wildtype and heterozygous mice had epididymal sperm numbers within the normal range (mean of 4.72×10^6 sperm per mouse).

Examination of seminiferous tubule cytology in adult males demonstrated a severe disruption of spermatogenesis in *msh5* -/- males compared to the normal abundance of all stages of the spermatogenic wave in wildtype males (Fig. 4B,C) and this was the cause of the reduction in testis size and absence of epididymal spermatozoa. In the mutant adult testis, interstitial Leydig cells and tubular Sertoli cells are present, as are type A and B spermatogonia (Fig. 4D,E) but, in contrast to wildtype testes (Fig. 4D), no spermatocytes are observed beyond zygotene (Fig. 4E). Immunohistochemical staining with anti-germ cell nuclear antigen 1 (GCNA1) revealed positive cells in the *msh5* -/- testis confirming the presence of germ cells whose position and size are indicative of early type A and B spermatogonia and pre-pachytene spermatocytes (Fig. 4G). In contrast, wild type males display a complete complement of spermatogenic cells including cells at pachytene, metaphase I, meiosis II, differentiating spermatids and fully differentiated spermatozoa (Fig. 4F).

In mice, the onset of prophase I of the first wave of spermatogenenesis commences at day 13 pp and is complete by day 25 pp, allowing the identification of discrete stages of meiotic progression. At day 17 pp, the seminiferous epithelium of wildtype males is densly packed with proliferating germ cells and with spermatocytes up to mid-pachytene (Fig. 5A; Bellve, 1977). In *msh5* -/- males, the seminisferous tubules are also fairly densly packed, although early signs of germ cell loss are evident, both by reduced GCNA1 positive cells and by the increase in apoptotic (TUNEL-positive) cells relative to that seen in wildtype tubules (Fig. 5B,C,D). By day 23 pp, the tubules of wildtype mice have progressed through an entire meiotic cycle and round spermatids have become evident by the loss of GCNA1 signal (Fig. 5E). In constrast, the tubules of *msh5* -/- males have lost significant mubers of germ cells (Fig. 5F) due to the sustained elevated level of apoptosis relative to that in wildtype seminiferous tubules (Fig. 5G,H). Thus, by 6 weeks of age, wildtype testes contain seminferous tubules packed with all stages of spermatogenesis (Fig. 5I) while the seminifours tubules of *msh5* -/- males have lost most of their cellular components beyond the level of the spermatogonia (Fig. 5J). The majority of remaining spermatocytes in the adult *msh5* -/- males are undergoing apoptosis (Fig. 5L). Thus, in the absence of *msh5*, there is a rapid and depletion of meiotic cells during the first wave of meiosis. Cells continue to be lost from the seminiferous tubules of *msh5* -/- males with each successive spermatogenic wave such that, by adulthood, almost the eintire spermogenic cell population is lost.

Meiotic arrest in *msh5* -/- males. The loss of spermatocytes from the first spermatogenic wave onwards in *msh5* -/- males suggests that there is a failure of

meiosis during early prophase I in these mice. Therefore, in order to analyse meiotic progession in further detail, meiotic chromosome spreads were prepared and studied at the light and electron microscope level. Chromosomes were prepared from testicular cell suspensions of 23 day old males to minimize the numbers of spermatids and spermatozoa present and to maximize the numbers of spermatocytes retrieved. In wildtype spreads, silver staining revealed a range of chromosomal configurations, including those at leptotene, zygotene, pachytene and diplotene. Chromosomes at leptotene and zygotene were unpaired, but at the latter stages of zygotene were beginning to condense and showed early signs of pairing. By pachytene, homologous chromosomes were synapsed along their entire lengths and were more fully condensed (Fig. 6A). In contrast, chromosome preparations from *msh5* -/- testes at day 23 pp did not contain any synapsed chromosomes although the chromosomes appeared to have replicated **to produce** sister chromatids (Fig. 6B), and all the spermatocytes examined appeared to be at the leptotene/zygotene stages of meiosis with only minimal condensation of the univalent chromosomes (Fig. 6B).

In order to determine whether the meiotic program is initiated in spermatocytes from *msh5* -/- males, we examined the chromosomal association of three proteins known to be required for recombination and formation of the synaptonemal complex (SC). The first two, SYCP1 and SYCP3, form integral components of the SC. SYCP1 (detected using the anti-syn1 antibody) is an integral component of the transverse filaments of the SC and is expressed from the zygotene to diplotene stages of prophase 1. SYCP3 (detected using the anti-cor1 antibody) is present on unpaired chromosome

axial cores prior to synapsis and is expressed from leptotene stages onwards. Immunofluorescant localization of these proteins on meiotic chromosomes using a combined antisera demonstrated normal acquisition of SC in spermatocytes from wildtype males and identified pachytene spermatocytes as having 20 distinct condensed pairs of bivalents (Fig. 6C). In *msh5* -/- spermatocytes, however, no bivalents were observed, but univalent chromosomes were clearly associated with the SYCP1/SYCP3 signal, indicating that axial element formation has been achieved and suggesting progression of meiosis at least through until mid-zygotene (Fig. 6D).

Rad51, a mammalian homolog of the bacterial RecA protein, is thought to participate in heteroduplex formation and/or chromosome repair following double strand break formation, and is an integral component of recombination nodules in lilies and possibly in mice. In wildtype spermatocytes, discrete Rad51 foci are located along the entire length of chromosomes during leptotene (Fig. 6E) and decrease steadily from zygotene through to pachytene as chromosomes become fully paired. In msh5 -/- spermatocytes, Rad51 is also localized in discrete foci along the univalent chromosomes (Fig. 6F) and, indeed, the number and intensity of these foci appears greater in the majority of msh5 -/- cells than on leptotene or zygotene chromosomes from wildtype males. Thus, the localization of Rad51 foci on the meiotic chromosomes from msh5 -/- spermatocytes does not decline, concurrent with the failure of synapsis in these cells.

Msh5 -/- females show a loss of oocytes and ovarian degeneration throughout life. To examine the role of **Msh5** in female meiosis, ovarian function was assessed in msh5 -/- adults. Msh5 -/- adult females did not mate with wildtype males, nor did they exhibit

characteristic estrous cycles. Gross anatomical observations indicated that the msh5 -/- females have normal oviducts, normally structured but hypotrophic uteri but lack discernible ovarian structures (Fig. 7D,E). Instead, the ovarian bursa of msh5 -/- females was empty or more frequently contained only a single cyst-like structure or a grouping of 3-4 cysts (Fig. 7E). To assess whether the absence of ovarian structure was due to developmental impairment or due to post-developmental degeneration of the ovary, ovaries from wildtype and msh5 -/- females were examined at day 3 pp and day 25 pp. At day 3 pp, there was no difference in ovarian size between wildtype and msh5 -/- females (Fig. 7A,B), although the ovaries of msh5 -/- females contained apparently fewer oocytes, as assessed by GCNA1 localization using an anti-GCNA antibody (Fig. 7B). By day 25 pp, the ovaries of msh5 -/- females were reduced to a small grouping of 1-3 follicles which appeared to be at post-antral stages of development, and occasionally contained oocytes (Fig. 7C). Wildtype ovaries at the same stage are similar to adult ovaries but with fewer corpora lutea and an abundance of primordial follicles (not shown). The presence of oocytes in the day 25 pp msh5 -/- females was confirmed by RT-PCR detection of transcripts for the oocyte-specific protein, zona pellucida 3 (ZP3) at day 25 pp in both wildtype and homozygous mutant females, However, ZP3 transcripts could only be detected in wildtype ovaries at adulthood (Fig. 7F). Thus, the ovaries of msh5 -/- females are normal size at birth, but then degenerate progressively to become rudimentary by adulthood. This ovarian degeneration is concomitant with a decline in oocyte numbers from before day 3 pp until adulthood, when no oocytes are present.

Msh5 -/- oogonia are meiotically incompetent and are almost entirely lost within the first 6 days of life. To examine the cause of the lower oocyte numbers in msh5 -/- females on day 3 pp, ovarian histogenesis was analyzed in female embryos from heterozygote mothers. Embryonic ovaries were removed from wildtype and *msh5* -/- females between embryonic day 15 (e15) and day 6pp. Alkaline phosphatase staining of e15 ovaries revealed normal acquisition of germ cells into the genital ridge in both female and male embryos (data not shown). Pre-meiotic and meiotic oocytes were then tracked using the anti-GCNA1 antisera between e16 and d6pp. At the early stages (e18 until day 1pp), the ovaries of homozygous mutant females contain apparently normal numbers of oocytes compared to wildtype (Fig. 8A,C). However, closer examination of H&E stained sections revealed subtle differences in chromosome structure between wildtype and msh5 -/- oocytes, characterized by clumping of nuclear contents in the homozygous mutant oocytes (Fig. 8D) compared to readily identifiable chromosomes in the wildtype oocytes (Fig. 8B). By day 3 pp, the number of oocytes present in the ovaries of msh5 -/- females was dramatically lower than that in wildtype ovaries (Fig. 8E,G), with more distinct morphological differences being notable; many oocytes from wildtype mice had grown considerably in size and were beginning to become encapsulated within primordial follicles, at the same time losing their anti-GCNA1 staining (Fig. 8F). In contrast, the ovaries of homozygous mutant females fail to undergo the expected architectural changes characteristic of early folliculogenesis, with oocytes remaining small and their nuclei retaining their dense staining with anti-GCNA1 antibody (Fig. 8H). By day 6 pp, large, well-formed primordial follicles

containing readily identifiable oocytes were distributed throughout the ovary of wildtype females (Fig. 8I, J), while in ovaries from msh5 -/- females the oocyte pool was completely diminished (Fig. 8K,L), with few or no GCNA1-positive oocytes being evident. Thus, in the absence of msh5, the ovaries become rapidly depleted of oocytes within days of the initiation of meiosis. Interestingly, the somatic tissues of the ovary fails to reorganize and to initiate folliculogenesis on day 2 pp as expected, despite the fact that the oocytes are not lost until after day 3 pp and this leads to the progressive loss of ovarian structure as described above.

DISCUSSION

Eukaryotes have a number of genes which are homologous to MutS. Of these, MSH2, MSH3 and MSH6 are ubiquitously expressed and form MSH2-MSH3 or MSH2-MSH6 complexes which are important for recognition of mispaired bases during mismatch repair. In yeast, MSH4 and MSH5 have distinct functions because mutations in these genes do not seem to affect mismatch repair but do affect meiosis resulting in reduced crossing over and reduced spore viability consistent with a defect in chromosome segregation. In mice and humans, MSH4 and MSH5 genes have more limited patterns of expression with maximal expression of MSH5 being found in gonads and lymphoid tissues. To assess the role of MSH5, we generated mice which have a mutation in this gene. There are two lines of evidence that suggest that the mutation is a null. We have introduced a hygromycin resistance cassette into exon 16 of the MSH5 gene disrupting an exon common to all alternatively spliced MSH5 transcripts. If transcripts are made from this modified locus, the translated product will not contain

the nucleotide binding domain and the helix-loop-helix domain located at the COOH-end that are important for the function of this family of proteins. Northern blot analysis of testis RNA from MSH5 -/- mice showed no MSH5 RNA. Our ability to detect MSH4 transcripts in these blots provided confidence that the RNA is derived from the appropriate source. Western-blot analysis clearly showed that the COOH end of the protein is absent. It is of interest to note that the levels of MSH4 RNA are reduced in testicular RNA. This could be explained by the depletion of germ cells.

The most striking phenotype in MSH5 deficient mice is that they are sterile consistent with **some** type of meiotic defect. This phenotype was detected in both sexes. The mice appear normal and males mate normally with wild-type females with vaginal plugs being detected after mating. The testes in adult mice are quite small and no mature spermatozoa were detectable in the epididymys or the semineterous tubules. There is a progressive loss of cells in the tubules and some of the tubules had no detectable cells in the adult. In mice, meiosis in males starts at day 14 pp. Prior to the initiation of meiosis, the tubules of MSH5 -/- mice are full of cells and were undistinguishable from +/+ mice. These results show that germ cell migration and the development of testes proceeds normally. The fact that Sertoli cells seem to be unaffected suggests that the intratubular environment is normal.

The failure of spermatagonial maturation is the result of abnormal meiosis. At day 17, wild-type males show all stages of meiosis along with spermatids and spermatozoa. In MSH5 -/- mice, however, only cells in leptolene-zygotene stages of meiosis I were detectable. Although occasional partially paired chromosomes were

detectable, there was a profound lack of chromosome pairing. What is remarkable is that the unpaired chromosomes were found to be associated with COR1 and SYN1, two proteins that are known to be components of synaptonemal complex, and Rad51, a homolog of the bacterial strand transfer protein RecA. In the absence of significant chromosome pairing, the meiotic cells do not appear to progress through meiosis but rather apotose. This meiotic phenotype of mice lacking MSH5 is different from that suggested by studies of MSH4 and MSH5 in S. cerevisiae. MSH4 and MSH5 function as a complex in meiosis and mutations in MSH4 and MSH5 cause a reduction in crossing over but not gene conversion during meiotic recombination. **Sporulation in these mutants occurs with reduced spore viability** which seems to result from chromosome non-disjunction due to the reduced levels of crossing over (**Ross-MacDonald et al, 1994**; Hollingsworth et al., 1995). The sporulation that occurs at reduced levels of crossing over that occurs in S. cerevisiae MSH5 mutants is in contrast to the lack of meiotic progression and apotosis that is observed in conjunction with greatly reduced meiotic chromosome pairing seen in MSH5 mutant male mice.

Crossing over is required for accurate chromosome seggregation during meiosis. Meiotic recombination appears to initiate due to the programed formation of double strand breaks in DNA. These double-strand-breaks have generally been thought to be repaired by classical double-strand-break repair mechanisms resulting in both gene conversion and crossing over **(Szostak et al, 1983)**. It has been suggested that MSH4 and MSH5 are required for correct processing of Holliday junctions during such double-strand-break repair to yield cross overs consistent with the reduction of crossing

over but not gene conversion in MSH4 and MSH5 mutants. Recently a variation of double-strand-break repair models of recombination calles synthesis dependent annealing recombination has proposed to be more consistent with the properties of meiotic recombination than classical double-strand-break repair **(Nassif et al, 1994; Paques et al, 1998)**. In this model, the 3' single stranded end present at one end of a double-strand-break invades the intact homologue and primes DNA synthesis thus copying information from the intact molecule onto one end of the broken molecule. This structure can them be processed in two ways: unwinding and annealing with the other broken end resulting in gene conversion and no crossing over; or conversion to the classical type of double-strand-break repair intermediate containing a double Holliday junction **allowing** both gene conversion and crossing over. If MSH4 and MSH5 were required for the formation of Holliday junctions during synthesis dependent annealing, a defect in this process would yield the lack of extensive chromosome pairing seen in MSH5 mutant mice and the reduced crossing over seem in S. cerevisiae MSH4 and MSH5 mutants. Such a defect in meiosis might lead to apotosis in mammalian cells and partial meiotic arrest, reduced sporulation and reduced spore viability in S. cerevisiae since S. cerevisiae lack apotosis.

Male mice which are deficient in PMS2 and MLH1 are also sterile like the MSH5 deficient mice but the phenotypes observed in each of these three mutant mice is different. Although MLH1 and PMS2 are members of a dimeric complexe that functions in somatic cells, PMS2 -/- mice show abnormal chromosome pairing and spermatogenesis; spermatids as well as spermatozoa, albeit abnormal in appearance,

were detected (Baker et al., 1995). In MLH1 mutant mice, the chromosomes pair but fail to proceed beyond pachytene (Edelmann et al., 1996; Baker et al., 1996). The homology relationships between MSH5 and the MSH proteins that interact with the MLH1-PMS2 heterodimer during mismatch repair suggests the possibility that MSH5 might interact with a MLH1 containing complex in meiosis (Edelmann et al., 1996). However, the different stages of meiosis where abnormalities are detected in MLH1, PMS2 and MSH5 mutant mice **suggest** that the role of these proteins in meiosis may be more complex that the simple model of a MSH4-MSH5 complex and a MLH1-PMS2 complex interacting with each other at the same stage in meiosis. For example the greater severity of the MLH1 mutant phenotype compared to the PMS2 mutant phenotype could reflect the possibility that PMS2 is partially redundant with regards to another MutL related protein such as the recently observed partial redundance between S. cerevisiae PMS1 (PMS2 in humans and mice) and MLH3 **(Flores-Rozas and Kolodner, 1998)**. In addition, the difference in the chromosome pairing defects in the MLH1 and MSH5 mutant mice suggests that these proteins may act at different stages of the homologous pairing process during meiotic recombination.

MSH5 -/- females exhibited the most dramatic phenotype. They were not only sterile but had a progressive degeneration of the ovaries. The lack of ovaries in the adult mice undoubtedly results in hormonal imbalance. The somewhat smaller size of the females that we observed could be attributed to this hormonal imbalance. Although we do not have direct evidence, the failure of the oocytes to proceed through meiosis I suggests that it is due to the lack of pairing of meiotic chromosomes. In normal mice,

pairing of homologous chromosomes is initiated, but the oocytes are arrested at this stage and hormonal stimulation results in the resumption of meiosis. Our results show that the absence of pairing has very profound effects on oocyte development. Oocytes seem to have a checkpoint control prior to the meiotic arrest. In the absence of pairing, the oocytes degenerate. The rapid loss of all oocytes and the attrition of the ovary suggest that pairing is a prerequisite for normal development and maturation of oocytes.

Meiotic abnormalities in the mutant mice. Both male and female msh5-/- mice are infertile as a direct result of a failure in gametogenesis. Males appear normal and exhibit normal reproductive behavior in the presence of wild type females and vaginal plugs were deteced after mating. The sterility in the males is due to the complete absence of spermatozoa either withing the seminfierous tubules or the epididymides. Developmental and morphological analysis of spermatogenesis in msh5 nullizygous males showed normal germ cell migration to the testis, but from day 14 pp, coincident with the onset of prophase I, there is a progressive loss of cells within the seminiferous tubules from the early spermatocyte stage. Despite this loss, seminferous tubule morphology is grossly maintained, with Sertoli cells present and a basal layer of type A and B spermatogonia. In the adult many sections of tubules are completely devoid of cells and the testis size is approximately 30% of wildtype.

The failure of spermatogonial maturation is the result of disrupted meiosis. At day 17 pp, wild type males exhibit cells at all stages of meiosis I within the seminferous tubules. In msh5 -/- males, however, only cells in leptotene to zygotene stages of

meosis I are detected and normal chromosomal pairing is never observed. During the first spermatogenic cycle, this failure of pairing triggers a checkpoint that results in a wave of apoptosis that removes all post-zygotene cells. With each subsequent cycle, the apoptosis continues, resulting in fewer and fewer cells within the tubules and the small adult testis size. Although most chromosomes do not pair, a very small proportion (less than 0.5%) show some degree of synapsis over short lengths of the chromosome though not necessarily involving homologous interactions. Interestingly, the localization of SYCP1 and SYCP3 on the unsynapsed chromosomes suggests that axial elements are constructed in the absence of Msh5 but that the tripartite synaptonemal complex, consisting of 2 homologous axial element-associated chromosomes linked together by a central core element, does not form.

Little is known about the onset of pairing and recombination and their relationship in mammals. In *S. cerevisiae*, the onset of recombination involves the formation of recombination nodules and double strand breaks (dsb), triggered by a complex of proteins including Spo11, Mre11 and Rad50 (Nairz and Klein, 1997), heteroduplex formation and strand exchange. These events preceed, and might be essential for, homologous pairing. In Drosophila, on the other hand, homologous pairing preceeds dsb formation (McKim *et al*, 1998) and other recombination events. In mammals, much of the recent data point towards a mechanism similar to that seen in yeast since proteins known to be involved in dsb formation and repair/stabilization, such as XRCC2, Rad51 and Dmc1 (Cartwright *et al*, 1998; Plug *et* al, 1996; Yoshida *et al*, 1998), are localized in discrete foci (recombination nodules) on unsynapsed meiotic

chromosomes. In msh5 mutant mice, Rad51 is localized on unsynapsed chromosomes **consistent with** the formation of early recombination nodules. This **Rad51 localization** is similar to that seen in Dmc1 mutant mice (Pittman *et al*, 1998), which also block at zygotene (Pittman et al, 1998: Yoshida *et al*, 1998), and suggests that double strand break formation proceeds normally in the absence of **Msh5**. Interestingly, meiotic chromosomes from msh5 -/- spermatocytes also show an increase in Rad51 foci, similar to that seen in dmc1 -/- spermatocytes, compared to that seen on chromosomes from wildtype spermatocytes at zygotene. **However, the lack of extensive chromosome pairing in mice lacking MSH5 suggests** that in the absence of **Msh5** in mice, meiotic crossing over is not initiated and that a complex including **Msh5** is an essential prerequisite for either the initiation and/or maintenance of a synapsed state. **As discussed above, the lack of chromosome pairing in MSH5 mutant mice and the reduced level of crossing over in S. cerevisiae msh5 strains is consistent with the view that Msh5 might act early in recombination to help produce stable, Holliday junction containing recombination intermediates.**

In adult msh5 -/- females, the phenotype is even more dramatic than in males becuase of the complete loss of ovarian structures. Consequently the females do not cycle and are smaller in size, probably as a result of a failure of ovarian steroidogenesis. The disruption of ovarian morphology is evident as early as day 3 pp, even though ovarian size is normal at this stage. Similar to the msh5 -/- males, the germ cells populate the genital ridge but as they progress through prophase I morphological analysis shows that there is widespread chromosomal disruption and the oocytes never

progress beyond zygotene. Thus, the loss of oocytes appears to be result from the failure of synapsis and the activation of a checkpoint resulting in apoptosis, as seen in msh5 -/- spermatocytes, This results in a progressive loss of oocytes from e18. By day 6 pp, there is an almost complete absence of oocytes and the ovary begins to degenerate such that, in the adult, it is usually entirely absent or consists of a few large cysts. The degenerating oocytes fail to initiate folliculogenesis showing that there must be dialog between the oocyte and the surrounding stroma for this process and, in turn, to maintain ovarian morphology.

The phenotype of the msh5 -/- females is remarkably similar to that seen in Turner's syndrome patients. In those patients with a complete loss of one X chromosome, there is acquisition of germ cells into the genital ridge which is followed by a rapid attrition of primary oocytes during the latter part of intrauterine and early neonatal life. This results in adults that have complete gonadal disgenesis, characterized by ÒstreakÓ gonads. It has been suggested that the cause of this degeneration is due to the loss of oocytes as a consequence of the failure of the single X chromosome to pair at zygotene (Singh and Carr 1966). The similarity between the msh5 phenotype and Turner's syndrome patients suggests that this failure of homologous pairing results in the triggering of an apoptotic checkpoint similar to that seen in the msh5 -/- oocytes, resulting in the complete loss of oocytes and ovaries.

EXPERIMENTAL PROCEDURE

Mouse MSH5 cDNA cloning. A segment of the mouse MSH5 gene was obtained by PCR using BALB/c genomic DNA (Clontech) and primers

GTGCTGTGGAATTCAGGATAC (sense) and CCAGAACTCTCTGGAGAAGC (antisense) based on the human cDNA sequence. PCR reactions (25 µl) contained 25 ng genomic DNA template, 0.2 μM each primer, 100 μM each dNTP, 0.75 U Amplitaq polymerase (Perkin-Elmer), 1.5 mM $MgCl_2$ in 10 mM Tris-HCl (pH 8.3), 50 mM KCl. Cycling parameters were 95¡C 5 min, 80¡C 4 min (1 cycle); 95¡C 30 s, 50¡C 15 s, 72¡C 30 s (35 cycles); with a final 5 min extension at 72¡C. **The resulting 118 bp product was purified by agarose gel electrophoresis using the QIAquick Gel Extraction Kit and sequenced directly using the same primers.** The 5' end of the cDNA was cloned by 5' RACE from mouse testis RNA (Clontech) using the 5' RACE Kit (BRL) with modifications of the manufacturer's recommendations. Gene specific primers used for the 5' RACE procedure were CCAGAACTCTCTGGAGAAGC (reverse transcription primer), TGGAGAAGCTTTAGGCTCTCGTGG (first-round amplification primer), and GCATCTGGCATGAAGTGGATAGTGGAG (nested amplification primer). Five-hundred nanograms of poly $(A)^+$ RNA was reverse transcribed in a 25 μl reaction according to the manufacturer's protocol. First strand cDNA was purified using QIAquick PCR Purification Kit (Qiagen) and eluted in 50 μl HPLC-grade water. Aliquots (10 μl) of purified cDNA were used as template in tailing reactions. Five microliters of each tailing reaction was used as template in 50 μl PCR reactions containing 0.2 μM each primer, 100 μM each dNTP, 1.5 U Amplitaq polymerase (Perkin-Elmer), 1.5 mM $MgCl_2$ in 10 mM Tris-HCl (pH 8.3), 50 mM KCl. Following electrophoresis in 1.5% agarose, 0.5? TBE gels, first-round products were purified using the QIAquick Gel Extraction Kit and eluted in 50 μl HPLC-grade water. Purified first-

round products were diluted 1/100 and 2 μl aliquots were used as template for second-round amplification with nested primers. Cycling parameters for both first-round and nested amplifications were 94¡C 5 min, 80¡C 2 min (1 cycle); 94 ¡C 30 s, 57¡C 30 s, 72¡C 30 s (35 cycles); with a final 7 min extension at 72¡C. Products of nested amplifications were purified, cloned and analyzed as described **for human MSH5 RACE products (Winand et al, 1998)**. The remainder of the mouse MSH5 coding sequence was cloned by RT-PCR using the Advantage cDNA PCR Kit and gene-specific primers CTCCACTATCCACTTCATGCCAGATGC (sense) and GCTGGGGAGGACACTGGAA-GGACTCTCA (antisense, based on human 3' untranslated cDNA sequence). Aliquots (5 μl) of cDNA prepared as described, using the 5' RACE Kit (BRL), were amplified in 50 μl PCR reactions containing 0.2 μM each primer, 200 μM each dNTP, 1 μl 50? Advantage KlenTaq polymerase mix in 40 mM tricine-KOH (pH 9.2), 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 75 μg/ml bovine serum albumin. Cycling parameters were 94¡C 1 min (1 cycle); 94¡C 30 s, 55¡C 30 s, 68¡C 4 min (35 cycles) followed by a final 7 min extension at 68¡C. PCR products were purified, cloned and analyzed as described for RACE products.

The mouse MSH5 genomic locus was cloned from a P1 mouse embryonic stem cell genomic library screened by Genome Systems, Inc. A single round of PCR screening with primers GTGCTGTGGAATTCAGGATAC (sense) and CCAGAACTCTCTGGAGAAGC (antisense) yielded three clones 11051, 11052, and 11053. Methods for the preparation of plasmid DNA and the analysis of the mouse

MSH5 intronic sequences **by direct sequencing of the P1 clones** were as described for the human MSH5 gene **(Winand et al, 1998)**.

Construction of the pMSH5ex16 targeting vectors. A genomic *Msh5* fragment containing exon 16 was obtained by screening a mouse genomic Charon 35, 129/Ola phage library with a 334 bp PstI probe corresponding to the region between nucleotides 1524 and 1858 of the mouse *Msh5* cDNA. A 3.8 kb HindIII fragment containing exon 16 was subcloned into pBluescript SK+/- and the intron-exon structure of part of the gene was determined using sequencing and restriction mapping. A 2.0 kb BglII PGKhygro cassette was cloned into the AatII site at codon 528 in exon 16 using BglII/AatII adaptors. The resulting gene targeting clone was designated pMSH5ex16.

Electroporation of embryonic stem cells. The targeting vector pMSH5ex16 (50ug) was linearized at the single XhoI site and electroporated into 5.0×10^7 WW6 embryonic stem cells (Ioffe et al. 1995) and selected with hygromycin (110ug/ml) as described previously (Sirotkin et al., 1995). Colonies were picked after 10 days and their DNA was screened by PCR using forward primer A 5'-AGCTGGAGAACCTGGACTCTC -3' and reverse primer B 5'-TGGAAGGATTGGAGCTACGG-3'. The reaction was performed in a 50 ul reaction mixture containing 100ng DNA, 5ng/ml of each primer, 1.75 mM $MgCl_2$, 0.3 mM of each dNTP and 2.8 U Expand Long Template enzyme mix.(Boehringer Mannheim). Cycling conditions were: 2 min at 94°C (1 cycle), 10 sec at 94°C, 30 sec at 60°C, 3 min at 68°C (10 cycles), 10 sec at 94°C, 30 sec at 60°C, 3 min (plus 20 sec extension/cycle) at 68°C (30 cycles) and 5 min at 68°C (1 cycle). Positive ES

cell colonies were identified by a 1.5 kb PCR fragment specific for the targeting event. Six positive cell lines MSH5-1, MSH5-33, MSH5-41, MSH5-52, MSH5-58, and MSH5-109 were identified and the correct targeting event was shown by NsiI digestion of high molecular weight DNA and Southern Blot analysis using a 0.8kb EcoRI/HindIII probe directed at the 5′ intron region between exons13 and 14 that is not included in the targeting vector.

Generation of MSH5 deficient mice. Chimeric mice were generated by injecting C57Bl/6 blastocysts with 8 to 12 embryonic stem cells derived from the MSH5-1 and MSH5- 52 colonies. The two cell lines gave rise to male chimeric animals which were mated with C57Bl/6 females. Chimeras obtained from both cell lines transmitted the *Msh5* mutation through the germline. F1 heterozygotes were interbred to obtain homozygous *Msh5* mutant animals.

Northern Blot Analysis. Total RNA from testis of 24 day old males was extracted and mRNA was isolated using oligo(dT)-cellulose columns (Stratagene mRNA kit). 4 ug of polyA RNA was separated on 1.0% Agarose Formaldehyde gels and transferred onto Nitrocellulose membrane. The membrane was subsequently hybridized with an Msh5 probe corresponding to exons 3 to 8, a probe spanning the complete mouse Msh4 cDNA and a human _-actin probe using the Strip-EZ kit (Ambion).

Western blot analysis. For Western blot analysis equal amounts of protein from testes extracts of 23 day old males were separated on a 10% SDS-polyacrylamide gel and transferred onto a Immobilon-P (Millipore) membrane. The membrane was blocked in TBS, 0.1% Tween-20, 5% nonfat dry milk, 10% goat serum (Sigma) and incubated with

1:1000 diluted primary anti-MSH5 antibody (Palambo et al., 1995). Bound protein was detected by chemiluminescence using a 1:300000 diluted goat anti-mouse IgG horseradish peroxidase conjugate (Sigma).

Histology. Ovaries from *msh5* +/+ and *msh5* -/- females between e18 and 5 wks postpartum (pp) were removed and fixed in Bouins or 4% buffered formalin for 30-360 minutes before transferring to 70% ethanol. Testes were fixed by transcardiac perfusion of 4% buffered formalin and then overnight in fresh fixative. All tissues were processed for histology by routine methods and were sectioned at 3 or 5 ?m.

Chromosomes. Chromosome spreads were prepared according to the method of Counce and Meyer (1973), with modifications. Spreads were then either silver stained in 50% silver nitrate at 65¡C for 6 hours (for electron microscopy) or subjected to immunofluorescence localization of chromosomally-associated proteins, according to the method of Moens and co-workers (Spyropoulos and Moens, 1994). Primary antibodies used were: (1) combined rabbit anti-syn1 and anti-cor1 antibody which detect the synaptonemal complex proteins, SYCP1 and SYCP3, contained within lateral and axial elements of the synaptonemal complex (1:1000) and (2) rabbit anti-Rad51 (1:500). Secondary antibodies were Fab fractions of either FITC or TRITC labelled anti-rabbit IgG (1:1000). Immunofluorescant images were captured used an Olympus Provis Ax70 epifluorescence microscope connected to a cooled (-40¡C) charge-coupled device (Photometrics CH250) and analysed using Scanalytics Cellscan software (Fairfax, VA).

ACKNOWLEDGEMENTS

The authors are grateful to the following colleagues for generously providing antibodies and advice: Drs Peter Moens and Barbara Spyropoulos (York University, Ontario, Canada; anti-synaptonemal complex antibodies), and Dr George Enders (University of Kansas Medical Center, Kansas City, KA; anti-GCNA1 antibody)

This work is supported by NIH grants (CA44704), American Cancer Society ( ) and a Center grant to AECOM ( ).

REFERENCES

Counce, S.J. and Meyer, G.F. (1973) Differentiation of the synaptonemal complex and the kinetochore in Locust spermatocytes studied by whole mount electron microscopy. *Chromosoma* 44, 231-253.

Cartwright R., Tambini C.E., Simpson P.J. and Thacker J. (1998) The XRCC2 DNA repair gene from human and mouse encodes a novel member of the recA/RAD51 family. Nucleic Acids Res. *26*, 3084-3089

McKim K.S., Green-Marroquin B.L., Sekelsky J.J., Chin G., Steinberg C., Khodosh R. and Hawley R.S. (1998) Meiotic synapsis in the absence of recombination. Science 279, 876-878

Nairz K. and Klein F. (1997) *Mre11S* - a yeast mutation that blocks double-strand-break processing and permits homologous synapsis in meiosis. Genes Develop. *11*, 2272-2290

Pittman D.L., Cobb J., Schiementi K.J., Wilson L.A., Cooper D.M. Brignull E., Handel M.A. and Schiementi J.C. (1998) Meiotic prophase arrest with failure of chromosome synapsis in mice deficient for *Dmc1*, a germlin-specific RecA homolog. Molec. Cell *1*, 697-705

Plug A.W., Xu J., Reddy G., Golub E.I. and Ashley T. (1996) Presynaptic association of Rad51 protein with selected sites in meiotic chromatin. PNAS *93*, 5920-5924

Singh, R.P and Carr, D.H (1966) The anatomy and histology of XO human embryos and fetuses. Anat. Rec. 155, 369

Yoshida K., Kondoh G., Matsuda Y., Habu T., Nishimune Y. and Morita T. (1998) The mouse RecA-like gene Dmc1 is required for homologous chromosome synapsis during meiosis. Molec. Cell 1, 707-718

Spyropoulos, B. and Moens, P.B. (1994) *In situ* hybridization of meiotic prophase chromosomes. *Methods in Molecular Biology* 33, 131-139.

Figure Legends

Figure 4. Disruption of spermatogenesis in *msh5* -/- males. (A) mRNA expression of *msh5* in testes from wildtype males between the ages of 8 days and 29 days, and in adult wildtype and *msh5* -/- males. (B,C,D,E) H&E stained sections of adult testis from wildtype (B,D) and *msh5* -/- (C,E) males showing loss of spermatocytes beyond zygonema in msh5-deficient males. Le, Leydig cell; S, Sertoli cell; A, type A spermatogonia; B, type B spermatogonia; PL, pre-Leptotene; L, Leptotene spermatocyte; Z, Zygotene spermatocyte; P, Pachytene spermatocyte; RS, round spermatid; ES, elongated spermatid; Sp, spermatozoa. (F, G) Immunolocalization of germ cells using anti-GCNA1 antibody on sections from wildtype (F) and *msh5* -/- (G) testes from 29 day old males showing abundant spermatocytes, seprmatids and spermatozoa in wildtype testes and and few cells in the msh5-deficient testes. For B and C, scale bar = 100 ?m; for D - G, scale bar = 25 ?m.

Figure 5. Progressive depletion of germ cells in *msh5* -/- males during development. (A,B,E,F,I,J) Germ cell immunolocalization using the anti-GCNA1 antibody of testes from wildtype (C,G,K) and *msh5* -/- (D,H,L) males showing the rapid depletion of germ cells from day 17pp onwards in msh5-deficient mice. (C,D,G,H,K,L) TUNEL staining of testes from wildtype (A,E,I) and *msh5* -/- males (B,F,J) showing continuous apoptosis from day 17pp onwards.

Scale bar = 100 ?m

Figure 6. Disruption of meiosis prior to synapsis in *msh5* -/- spermatocytes. (A,B) silver-stained spermatocytes from wildtype (A) and *msh5* -/- (B) testes showing failure of pairing in the absence of msh5. (C,D) Immunofluorescant localization of SYCP1 and SYCP3 proteins on synaptonemal complexes of wildtype pachytene spermatocytes (C) and on axial elements of unsynapsed leptotene/zygotene spermatocytes from *msh5* -/- testes (D). (E,F) Immunoflourescant localization of Rad51 on leptotene spermatocytes from wildtype (E) and *msh5* -/- (F) males.

Figure 7. Loss of oocytes and subsequent ovarian degeneration in *msh5* -/- females. (A,B) Ovaries from day 3pp wildtype (A) and *msh5* -/- (C) females showing oocytes stained with GCNA1. (C) Entire ovary from a day 25pp msh5 -/- female (H&E staining) containing only 3 follicles and degenerating tissue. (D,E) Ovaries from adult wildtype (D) and msh5 -/- females (H&E stained) showing complete loss of oocytes and ovarian architecture in the absence of msh5. B, ovarian bursa;Ov, oviduct. In all cases, scale bar = 200 ?m. (F) Expression of ZP3 and Actin in ovaries of wildtype and msh5 -/- ovaries on day 25pp and in the adult.

Figure 8. Disruption of oogenesis in *msh5* -/- females leads to a failure of folliculogenesis. (A,B,C,D) Ovaries from e18 wildtype (A,B) and *msh5* -/- (C,D) embryos showing oogonia stained with GCNA1 (A,C) or H&E localization of meiotic chromosome detail (C,D). (E,F,G,H) GCNA1 localization of oocytes in ovaries from day

3pp wildtype (E,F) and *msh5* -/- (G,H) females. Arrowheads indicate pachytene oocytes (punctate red staining of nucleus compared to solid red staining of pre-pachytene oocytes), arrows indicate the appearance of the earliest primordial follicles. (I,J,K,L) GCNA1 localization of oocytes in ovaries from day 6pp wildtype (I,J) and *msh5* -/- (K,L) females. Arrows indicate primordial follicles; o, oocyte.For A,C,E,G,I, and K, scale bar = 100 ?m; for B,D,F,H,J,and L, scale bar = 25 ?m.